Regulation of Behavioral Arousal and Quiescence in C. elegans

Regulation of Behavioral Arousal and Quiescence in *C. elegans*

A dissertation presented

by

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to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological and Biomedical Sciences

Harvard University

Cambridge, Massachusetts

April 2016

Dissertation Advisor: Dr. Joshua Kaplan Kelsey Patricia Taylor

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Abstract

Animals switch between periods of behavioral quiescence and arousal in response to environmental, circadian, or developmental cues. *C. elegans* exhibit periods of behavioral quiescence during larval molts (termed lethargus) and as adults. Little is known about the circuit mechanisms that establish these quiescent states. Mutants lacking the neuropeptide receptor NPR-1 are a model for heightened arousal and have dramatically reduced locomotion quiescence during lethargus as a result of increased sensory acuity and secretion of the arousal peptide PDF-1.

In Chapter 2 of this thesis, we show that the aroused locomotion of *npr-1* mutants results from the exaggerated activity in multiple classes of sensory neurons, including nociceptive (ASH), touch sensitive (ALM and PLM), stretch sensing (DVA) neurons, and chemosensory neurons (ASI). These sensory neurons accelerate locomotion via both neuropeptide and glutamate release and their relative contribution to arousal differs between larval molts and adults. These results demonstrate that a broad network of sensory neurons and transmitters dictates transitions between aroused and quiescent behavioral states. We propose that locomotion quiescence during molts is mediated by diminished sensory inputs (termed sensory gating) and that NPR-1 plays a central role in this process.

In Chapter 3, we identify a second arousing neuropeptide, FLP-2, which promotes locomotion through an orexin-like receptor (FRPR-18). FLP-2 secretion is inhibited by NPR-1 and enhanced secretion is associated with aroused locomotion during molts. This locomotion arousal is stabilized by reciprocal positive feedback between two arousing neuropeptides (FLP-2 and PDF-1). FLP-2 and FRPR-18 are co-expressed in ASI neurons, suggesting that ASI activity is regulated by autocrine positive feedback. Our results suggest that FLP-2 and FRPR-18 are the *C. elegans* homologs of mammalian hypocretin/orexin peptide and receptor, respectively. We propose that aroused locomotion is stabilized by two circuit motifs: reciprocal positive feedback between different classes of arousing neurons and autocrine positive feedback of FLP-2 expressing neurons. These motifs may be conserved in the arousal circuits of other model systems.

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ACKNOWLEDGEMENTS

First, I would like to thank my advisor Josh Kaplan, who has been a wonderful advisor throughout graduate school. Joining his lab was the best decision I made as a student, and not once have I regretted it. Josh has supported me since day 1 in his lab. He is always willing to give me advice, whether it be on the fine details of an experiment, which projects to pursue (or sadly let go), career guidance, or how to best present my research in presentations. Not only has he supported my academic ventures, he has also put up with my other "projects" throughout the years. These have included: the Happiness Project, Mol Bio Run Club, and the birthday cake rotation, which after 4 years I finally discovered his birthday and added him to, despite his protests. Jokes aside, Josh is a brilliant scientist to whom I am very appreciative for everything he has taught me.

The members of the Kaplan Lab have become like family over the years, and I am forever grateful for their mentorship, friendship, and support. I couldn't have been luckier than to have Seungwon "Sebastian" Choi as a rotation mentor; he spent endless hours teaching me how to pick and identify "sleeping" worms while also conducting my first RNAi screen with me side by side. He was gracious enough to collaborate with me on the glutamate paper, and pass on the 'sleep project' after his graduation from the lab. Ed Pym has also been a tremendous teacher and friend to me since the beginning. Not only did he give me my infamous nickname (seriously does Bob Kingston even know my real name?), Sparkles, he also taught me how to make a cup of British tea, the pronunciation of aluminum, and the names of all 5 Beatles. Tongtong Zhao and I started in the lab at the same time, and I will miss commiserating with her at lunch, teatime, cake-time, and all events involving food. I have always admired Tongtong's perseverance in the lab, and its

something I hope to emulate in my own career. Xiajing Tong is a ray of sunshine in the lab, and I'm endlessly impressed with how much work she presents at each lab meeting. I think running busily around the lab is how she burns off all of those Starbucks frappuccinos. Stephen 'Nuz' Nurrish is a brilliant and badass scientist; I only wish I could have had more time learning from him. One day he, Ed, and The WOPR will take over the world. Thank you to Yingsong Hao and Qi for being mother figures to the lab, and to Amy Vashlishan-Murray for being a mentor and friend. Daniel Nedelcu, Hayley Mattison, Cindy Chang and Peter Hoboth are more recent additions to the Kaplan Lab who have each brought fresh personalities and spice to our community.

I also want to thank several previous members of the lab during my time here. Despite his crazy antics, I'm thankful to Dorian Anderson, who was one of my first mentors after joining the lab and was always willing to answer my questions. Didi Chen was my partner in crime on multiple projects; I'm grateful for her partnership through the FLP-2 project and for answering my many emails even all the way from Texas. Katie Thompson-Peer graduated soon after I joined the lab, but was someone I admired and looked to for advice on numerous occasions. Thank you also to Monica Feliu-Mojer and Tambu Kudze for bringing so much joy to the lab, Zhitao Hu for showing me that even super-humans take naps at work, and Nikhil Sasidharan for his endless positively and appreciation for people.

I would be remiss to not thank the entire MGH Molecular Biology Department. Surrounded by the Ausubel and Ruvkun labs, there were few to no times that I could not find someone to answer a worm-related question. In many ways, my colleagues on the floor felt like an extension of our own lab. Thank you also to the support staff: Joanne

Desmond, Stanton Young, Neil Hendersen for all their help over the years. I would also like to thank my Dissertation Advisory Committee (DAC), Lisa Goodrich, Max Heiman, and Beth Stevens, for making time in their schedules to consult on my work. They provided a fresh perspective that always helped guide my experiments in the right direction. In particular, Lisa has been a great support as a member of my prequalifying exam committee, chair of my DAC, and now as chair of my thesis exam committee.

Coming to Harvard likely wouldn't have been possible if not for the research experience I was afforded as an undergraduate; Thank you to my Connecticut College advisor, Bruce Branchini, for introducing me to science research in his lab, and Tom Schwarz for allowing me to intern in his lab with the Summer Honors Undergraduate Research Program (SHURP) at HMS in the summer of 2010. Thank you to the late Jocelyn Spragg and Sheila Thomas for accepting me into the program and giving me the opportunity to conduct research at a higher level.

Over the last 5 years my classmates, teachers, and advisors made graduate school feel like home. A huge thanks to Kate Hodgins, Maria Bollinger, and Danny Gonzalez in the BBS office for making the BBS program a community I'm proud to be part of. Genetics 201 will forever be one of my favorite classes and played a large role in steering me towards a genetics lab. Thank you to Fred Winston, Emily Gleason, and the entire Program in Genetics in Genomics for being another source of academic support I could rely on and learn from. Thank you also to my friends and colleagues at Science in the News and the Graduate Consulting Club who gave me additional outlets to explore my education and career trajectory.

I am profoundly thankful to my friends, who have kept me sane throughout these crazy years. I'd especially like to thank my fellow classmates, who best understand the grad school experience having been side-by-side with me on this journey since day one. I can always rely on them to let loose and have fun with, or have intense conversations about research and experiments. Of special note are my girls Erica Sennott and Naomi Olsen, and the guys Andrew Giacomelli, Jon Henninger, Chris 'Tiger' Davis, Kevin Harlen, and Brian Pepe-Mooney. Thank you also to my running friends at November Project and Fenspeed, who provided me with the athletic community I needed here in Boston and convinced me that running 4 marathons in my time at graduate school would be a reasonable and fun thing to do. Last, thank you to Danielle, Pamela, Kelsey A, and Haley for their lifelong friendships, and to Ben for jumping on the tail end of this journey and being so encouraging.

Finally, I deeply thank my family for supporting me throughout these years. I'm forever in debt for their support, encouragement, and general presence in my life. My parents are the two hardest working people I know and have provided me with continuous love and support. They might not recognize it, but I'd be lucky to be half the people they are someday.

Words can never describe how thankful I am for these humans. Every person listed here is not only a colleague and friend, but also an extension of my family. Thank you, thank you, thank you!

Chapter 1

Introduction

Circadian and homeostatic regulation of behavioral arousal in mammals

Animals switch between periods of behavioral arousal and quiescence in response to environmental, developmental, and circadian cues. While behavioral arousal is characterized by increased responsiveness to sensory stimuli and motor activity, quiescence is associated with decreased activity and responsiveness (Pfaff et al., 2008). The biological mechanism underlying behavioral arousal and quiescence is best studied in the sleep/wake cycle. Aside from quiescence (or lack of movement), an increased arousal threshold is the main behavioral feature distinguishing sleep from rest (Cirelli, 2009). The other accepted facets of sleep behavior include lack of reactivity to sensory inputs, rapid reversibility, and homeostatic response to sleep deprivation (Zimmerman et al., 2008).

For the last three decades, the two-process model of sleep regulation has been the dominant conceptual model for sleep in humans (Borbély et al., 2016). The model posits that sleep is regulated by two main factors, circadian rhythm and homeostatic pressure. Circadian rhythm considers the body's internal processes of alertness that is determined by the internal biological clock, promoting consolidation of sleep into one major phase. The circadian rhythm is independent of the amount of preceding sleep or wakefulness, typically following the 24 hour day-night cycle. In contrast, homeostatic mechanisms regulate the balance between sleep pressure and wakefulness, with homeostatic pressure increasing with time spent awake and vice versa. Thus, if wakefulness lasts for an extended period of time, homeostatic pressure increases and promotes sleep to compensate for sleep deprivation (Cirelli, 2009). In mammals, the suprachiasmatic nucleus (SCN) in the hypothalamus is responsible for circadian rhythms while multiple

brain regions contribute to homeostatic changes (Borbély and Tobler, 2011; Cirelli, 2009; Cirelli and Tononi, 2011). Disruption of either circadian or homeostatic regulation can cause fragmentation of sleep and various sleep disorders.

A variety of explanations for the purpose of sleep have been proposed regarding cellular and molecular aspects of biology. Sleep may function to allow time for neuronal synapse changes to occur (synaptic plasticity), to facilitate processes of learning and memory, to help restore brain energy stores that are depleted during wakefulness, or promote biosynthesis and recovery from cellular stress (Benington and Heller, 1995; Cirelli and Tononi, 2011; Mignot, 2008; Scharf et al., 2008). Indeed, analysis of expression profiles during sleep suggest that sleep isn't a global state of CNS inactivity, but may play a positive role in brain protein synthesis, maintenance, synaptic plasticity and membrane trafficking (Cirelli and Tononi, 2011). It has recently been proposed that function of sleep can be described in a single unifying theory, the Energy Allocation Model of Sleep. This model states that sleep behaviors provide all species the ability to optimally allocate energy utilization in order to maximize reproductive success while meeting environmental energy constraints (Schmidt, 2014).

Neuropeptide regulation of behavioral arousal

Through studies in humans, mice, *Drosophila*, *C. elegans*, and other model systems, it is well known that behavioral arousal is modulated by a complex variety of neurotransmitters. In mammals, the precise circuit mechanisms of how these neurotransmitters work together is not well understood. Here, I will briefly discuss the various neuropeptides known to modulate arousal in mammals including

hypocretin/orexin, NPY, growth hormone-releasing hormone (GHRH), and corticotropin-releasing factor (CRF). While I will focus on these, a multitude of other peptides have been shown to influence behavioral arousal, both endogenously and exogenously, including dopamine, glutamate, galanin, vasoactive intestinal polypeptide (VIP), and melanin-concentrating hormone (Kotronoulas et al., 2009). Some of these will be further discussed below in reference to their role in *C. elegans* quiescence and arousal.

Hypocretin/orexin signaling was first identified as being essential for stabilizing sleep and wakefulness in dogs. Canine narcolepsy is an autosomal-recessive, fully penetrant disorder due to mutation in hypocretin/orexin receptor 2 gene (Lin et al., 1999). In human narcolepsy, patients cannot maintain long waking periods, and experience abrupt transitions into non-REM (NREM) sleep and abnormal intrusions of REM sleep into waking (Cirelli, 2009). Similarly, dogs with narcolepsy experience fragmented sleeping patterns and episodes of cataplexy (or muscle atonia), typically following emotional excitement (Nishino and Mignot, 1997). In mice, a null mutation in prepro-hypocretin (orexin) peptide gene causes behavioral arrest and EEG patterns also reminiscent of narcolepsy (Chemelli et al., 1999; Cirelli and Tononi, 2011). In humans, narcolepsy is also associated with hypocretin/orexin deficiency (Nishino et al., 2000). While no association has been found between human narcolepsy and polymorphisms in the hypocretin/orexin genes, one case of early onset narcolepsy was found to be associated with mutation in the preprohypocretin gene (Peyron et al., 2000). Together, these studies suggest that hypocretin/orexin acts to promote wakefulness in animals as an 'arousal peptide'.

In contrast, a peptide known to inhibit arousal/wakefulness is Neuropeptide Y (NPY). NPY is broadly expressed in the human brain and is involved in diverse physiological functions including food intake, hormone release, thermoregulation, stress and anxiety (Dyzma et al., 2010; Thorsell and Heilig, 2002). During sleep, NPY is thought to promote sleep by inhibiting corticotropin-releasing hormone (CRH), a wake promoting peptide in humans. Administration of NPY results in increased sleep duration and decreased sleep latency and wake time (Antonijevic et al., 2000; Held et al., 2006). In *Drosophila*, the NPY homologue neuropeptide F (NPF), and its receptor NPFR1 promote sleep duration and quality (He et al., 2013). Together, these studies suggest that NPY inhibits behavioral arousal in mammals as a sleep-promoting, or somnogenic, peptide.

Another mammalian somnogenic peptide is growth hormone-releasing hormone (GHRH), which promotes NREM sleep and acts in opposition to the wake promoting peptide CRH. Increased levels of CRH, either by overexpression or administration to the CNS, have been shown to act on the hypothalamaic-pituitary-adrenal (HPA) axis to decrease sleep duration in rats, and even induce anxiety-like behavior in mice (Ehlers et al., 1997; Stenzel-Poore et al., 1994). Studies in humans suggest that the ratio between these two hormones is critical to the regulation of sleep and wakefulness (Kotronoulas et al., 2009).

While a large number of neurotransmitters and peptides have been identified to regulate sleep in humans and other mammals, very little is known about how these factors interact with one another in neural circuits to affect arousal. The organism *C. elegans* provides a powerful genetic model to study the neural and molecular basis of sleep and arousal.

Lethargus is a sleep-like state in *C. elegans*

During the four larval molts, *C. elegans* undergo a profound period of behavioral quiescence termed lethargus that persists for 2-3 hours. This behavior has properties of a sleep-like state including cessation of feeding and movement, decreased response to sensory stimuli, a stereotypical posture, and showing a homeostatic response to sleep deprivation (Iwanir et al., 2013; Raizen et al., 2008). While other behaviors in *C. elegans* have also been described as sleep-like, such as quiescence following stress or satiety (Gallagher et al., 2013; Hill et al., 2014; Jones and Candido, 1999; Nelson et al., 2014; You et al., 2008), this discussion will focus on the regulation of the molting-associated quiescent state, lethargus.

Despite the fact that *C. elegans* lethargus does not occur with a 24-hr circadian rhythm, many molecular pathways implicated in sleep in higher organisms have been found to similarly regulate *C. elegans* molting-associated quiescence. First, *C. elegans* molting and lethargus is regulated by a conserved molecular clock; the cycle is mediated by rhythmic changes in expression of a heterochronic gene, *lin-42*, which is homologous to the fly circadian gene PERIOD. *lin-42* mutants display abnormal timing of molting and lethargus, while forced expression of LIN-42 results in lethargy in adult animals (Monsalve et al., 2011). Additionally, a number of other pathways have been identified to regulate quiescence in *C. elegans* with conservation across sleep behaviors in other organisms, as summarized in Table 1.1.

Table 1.1 Conserved pathways in the regulation of behavioral quiescence

Pathway	Mammals	*Drosophila*	*C. elegans*
PERIOD	Humans and mice have three PERIOD homologues, Per1, Per2, Per3 which show rhythmic expression in the SCN and other brain regions. Mutations in period genes are linked to aberrant sleeping patterns and disorders (Ko and Takahashi, 2006; Tei et al., 1997)	Mutations in Period (per) gene affect circadian rhythm of flies, and per mRNA shows oscillation consistent with circadian cycle (Hardin et al., 1990)	*lin-42* (period) mutants display abnormal timing of molting and lethargus, while forced expression of LIN-42 causes lethargic behavior in adult animals (Monsalve et al., 2011)
PDF (Arousing)	VIP is expressed in the SCN of rats and mice lacking VIP exhibit reduced REM sleep (Hu et al., 2011; Maywood et al., 2007)	PDF mutants show defects in circadian timing and locomotive activity (Parisky et al., 2008; Renn et al., 1999)	Secretion of PDF-1 is decreased during lethargus (Choi et al., 2013)
NPY (Sleep promoting)	Administration of NPY promotes sleep in humans (Antonijevic et al., 2000; Held et al., 2006)	NPF and NPFR1 promote sleep duration and quality (He et al., 2013)	*npr-1* mutants show blocked quiescence during lethargus (Choi et al., 2013)
cAMP/PKA/ CREB (Arousing)	Mutations in cAMP-regulated binding protein (CREB) in mice have decreased cortical arousal and increased sleep (Graves, 2003)	cAMP signaling and CREB activity are inversely correlated with duration of sleep (Hendricks et al., 2001)	Mutants with increased PKA activity and cAMP levels show reduced quiescence in lethargus and increased responsiveness to sensory stimuli (Belfer et al., 2013; Iwanir et al., 2013; Raizen et al., 2008; Singh et al., 2014)

Table 1.1 (Continued)

Pathway	Mammals	*Drosophila*	*C. elegans*
Dopamine (Arousing)	Clinical drugs that increase dopamine release promote wakefulness (Wisor et al., 2001)	Local dopamine pathway promotes arousal/wakefulness in flies (Ueno et al., 2012)	Mutations in D1 receptor *dop-1* increase quiescence, while mutations in dopamine transporter *dat-1* reduces quiescence (Singh et al., 2014)
PKG (Sleep promoting)	Brain specific knockouts of PRKG1 reduces drive to sleep (Langmesser et al., 2009)	Decreased PKG activity in *foraging* mutants (PKG gene) show decreased sleep (Donlea et al., 2012)	Gain-of-function (GOF) mutants in *pkg-1* increase quiescence and arousal threshold in lethargus, while loss-of-function (LOF) mutants show the opposite phenotype (Raizen et al., 2008)
Serotonin (Sleep promoting)	Role of Serotonin in mammals is unclear, with studies showing both sleep-promoting and wake-promoting roles for serotonin (Ursin, 2002)	Loss of Drosophila serotonin receptor 5-HT1A decreases rest (Yuan et al., 2006)	Serotonin-like receptor *ser-4* mutants show decreased total quiescence in lethargus (Singh et al., 2014)

Some insights have been made into how these neuropeptides and transmitters regulate quiescence in *C. elegans* neural circuits. The glutamatergic RIA interneurons express the sleep-promoting peptide NLP-22, which inhibits feeding and locomotion during lethargus and shows cyclical mRNA expression in synchrony with molting/lethargus. However, activation of RIA inhibits quiescence during lethargus suggesting a more complex role of the interneuron in regulating arousal (Nelson et al.,

2013). In contrast, the GABAergic interneuron RIS has been identified as a sleep-promoting neuron that is active during lethargus and inhibits locomotion upon optogenic activation. This inhibition requires peptide synthesis, but not GABA synthesis, suggesting that a peptide from RIS is critical to promoting quiescence during lethargus (Turek et al., 2013). The major sleep-inducing neuropeptide in RIS was identified as FLP-11, which is released by depolarization of RIS at the onset of sleep. FLP-11 is constantly expressed in RIS (under the control of the transcription factor APTF-1) and can induce quiescence at anytime RIS is activated (Turek et al., 2016).

The circuitry controlling lethargus is at least partially distinct from adult forms of quiescence, such as that induced by stress in *C. elegans* (Trojanowski et al., 2015). For example, the peptidergic ALA neuron and secretion of the peptide FLP-13 are required for stress-induced quiescence, but is not required for molting-associated quiescence (Nelson et al., 2014). While these insights have been notable, further research on the neural basis of arousal in *C. elegans* will increase our understanding of the mechanisms behind sleep behaviors including decreased sensory responsiveness and gating, homeostasis, quiescence, and the role of sleep in health.

A role for NPR-1 in *C. elegans* sensory gating and arousal

A key regulator of dampening sensory responses in *C. elegans* is the neuropeptide-Y receptor *npr-1,* a NPY receptor homolog (de Bono and Bargmann, 1998). NPR-1 expression is concentrated in a central sensory circuit defined by gap junctions to the RMG interneuron (Fig. 1.1) where it acts to inhibit activity of the sensory neurons (Coates and de Bono, 2002; de Bono and Bargmann, 1998). Mutations inactivating *npr-1*

result in heightened activity in the central sensory circuit, which alters a variety of its behavioral outputs. For example, *npr-1* mutants exhibit exaggerated responses to oxygen, carbon dioxide, pheromone, and pathogen due to increased acuity of the RMG circuit. These heightened sensory responses are associated with exaggerated locomotion and for this reason, *npr-1* mutants can be used as a model for generalized arousal (Bretscher et al., 2008; Cheung et al., 2005; Gray et al., 2004; Hallem and Sternberg, 2008; Macosko et al., 2009; Reddy et al., 2009; Srinivasan et al., 2012; Styer et al., 2008).

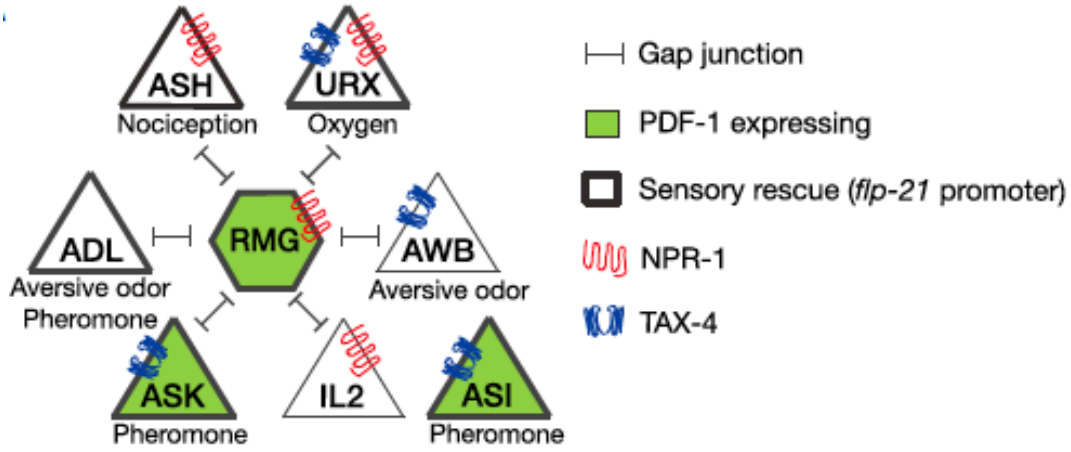

Figure 1.1 A schematic of the RMG circuit. Sensory neurons (triangles) mediating diverse responses form gap junctions with the central RMG interneuron (hexagon). Cells expressing NPR-1, TAX-4/CNG channels, PDF-1 and the *flp-21* promoter (sensory rescue) are indicated (Barrios et al., 2012; Coates and de Bono, 2002; Janssen et al., 2009; Komatsu et al.; Macosko et al., 2009; Rogers et al., 2003). ASI neurons are not directly connected to RMG but are also a potential source of PDF-1. This diagram is modified from previous work (Macosko et al., 2009) and was previously published (Choi et al., 2013) .

We previously found that in addition to these sensory behaviors, *npr-1* is also required for locomotion quiescence during lethargus. Locomotion quiescence during lethargus is nearly completely blocked in *npr-1* mutants. The quiescence defect can be restored by expression of *npr-1* in RMG and sensory neurons (*flp-21* promoter), and can be blocked by mutations inactivating ion channels required for sensory transduction, such as TAX-4/CNG channels (Fig. 1.1), suggesting that the *npr-1* quiescence defect requires increased RMG sensory activity (Choi et al., 2013). In microfluidic chambers where sensory cues are minimized, *npr-1* mutants only have modest defects in lethargus quiescence unless given a brief stimulation with light or vibration, suggesting that sensory activity is critical for the aroused locomotion (Nagy et al., 2014a). *npr-1* was also found to be required for homeostatic rebound following a weak disruption to quiescence during lethargus (Nagy et al., 2014b).

The arousing effects of *npr-1* mutation are mediated by increased secretion of the neuropeptide Pigment Dispersing Factor (PDF-1) from sensory neurons controlled by the RMG circuit. PDF-1 acts on PDF receptors (PDFR-1) expressed in the peripheral mechanosensory neurons to drive locomotion (Choi et al., 2013). The neuropeptide PDF was first implicated in *Drosophila* sleep, where it is expressed in central clock neurons (LNv neurons) and similarly promotes arousal; flies mutant for PDF or its receptor PDFR show defects in circadian timing and morning sleep (Parisky et al., 2008; Renn et al., 1999). *C. elegans* PDF-1 and *Drosophila* PDF are thought to be the functionally analogous to VIP, an arousal peptide expressed in the central clock SCN neurons in mammals (Choi et al., 2013; Hu et al., 2011; Maywood et al., 2007).

Dissertation overview

The experiments of this dissertation investigate the regulation of arousal and quiescence using the model organism *C. elegans* and aim to better understand how these behaviors are regulated by neuronal circuits, neurotransmitters, and sensory perception.

In chapter 2, I compare the circuits regulating arousal in larval molts and adults and describe a role for glutamate and AMPA receptors in this behavior. We show that a broad network of sensory neurons arouses locomotion but that the impact of each neuron differs between lethargus and adults. We propose that this broad sensory network allows *C. elegans* to adapt its behavior across a broad range of developmental and physiological circumstances.

In chapter 3, I describe the role of the neuropeptide FLP-2 in arousing locomotion during lethargus. We show that FLP-2 acts via an orexin-like receptor, FRPR-18, in sensory neurons in the worm. In addition, we demonstrate that aroused locomotion is mediated in part by concerted action of both FLP-2 and PDF-1, and is stabilized by reciprocal positive feedback between these two arousal peptides. We propose that FLP-2 and FRPR-18 are the *C. elegans* homologues of mammalian hypocretin/orexin peptide and receptor, respectively, and as such are the first hypocretin/orexin pathway identified in an invertebrate model system.

In chapter 4, I comment on the implication of our findings, with emphasis on the broad network of sensory neurons that contribute to arousal, understanding sensory gating as a mechanism for quiescence, and conservation across phylogeny. I also comment on preliminary results and future experiments regarding ascaroside, octopamine

and tyramine regulation of behavioral quiescence, the possibility of synaptic remodeling during lethargus, and the role of the RMG circuit in circadian timing.

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Chapter 2

Sensory Neurons Arouse *C. elegans* Locomotion via both Glutamate and Neuropeptide Release

This chapter contains work published as Choi S, Taylor KP, Chatzigeorgiou M, Hu Z, Schafer WR, Kaplan JM (2016) PLOS Genetics 11(7)

Author Contributions

Marios Chatzigeorgiou performed all of the ASH calcium recordings. Zhitao Hu contributed to some of the electrophysiology recordings. Kelsey Taylor and Seungwon Choi equally contributed to all of the other experiments. Kelsey Taylor, Seungwon Choi, and Joshua Kaplan assembled the manuscript, with input from William Schafer and Marios Chatzigeorgiou.

Acknowledgments

I would like to thank the following for strains, advice, reagents, and comments on the manuscript: Cori Bargmann for NPR-1 sensory rescue and rat TRPV1 transgenes, the *Caenorhabditis* Genetics Center (CGC) and S. Mitani for strains, and David Biron and members of the Kaplan lab for comments.

Introduction

Animals undergo periods of behavioral quiescence and arousal in response to changes in their environment and metabolic state. Arousal is defined as a state of heightened responsiveness to external stimuli coupled with increased motor activity whereas quiescence is associated with diminished responsiveness and motor activity (Pfaff et al., 2008). Quiescence and arousal can persist for minutes to hours. Arousal is associated with fear, stress, hunger, and exposure to sexual partners (Pfaff et al., 2008), while quiescence is associated with sleep and satiety (Cirelli, 2009). Relatively little is known about the specific circuit mechanisms leading to arousal or quiescence. In particular, it is unclear if similar mechanisms mediate quiescence and arousal in response to different cues, or at different times during development. To address this question, we have analyzed arousal and quiescence of *C. elegans* locomotion.

During each larval molt, *C.elegans* undergoes a prolonged period of profound behavioral quiescence, termed lethargus behavior, whereby locomotion and feeding behaviors are inactive for approximately 2 hours (Cassada and Russell, 1975). Lethargus has properties of a sleep-like state such as reduced sensory responsiveness and homeostatic rebound of quiescence following perturbation (Raizen et al., 2008). Several genes and molecular pathways involved in lethargus behavior have been identified (Choi et al., 2013; Monsalve et al., 2011; Nagy et al., 2013; Nelson et al., 2013; Raizen et al., 2008; Singh et al., 2011; Turek et al., 2013; Van Buskirk and Sternberg, 2007). Multiple sensory responses are diminished during lethargus, including those mediated by a nociceptive neuron (ASH) (Cho and Sternberg, 2014), and by mechanosensory neurons (Choi et al., 2013; Schwarz et al., 2011).

Mutants lacking NPR-1 Neuropeptide Y (NPY) receptors have been utilized as a model for generalized arousal. NPR-1 inhibits the activity of a central sensory circuit that is defined by gap junctions to the RMG interneuron (Macosko et al., 2009). In *npr-1* mutants, responses mediated by the RMG circuit (e.g. pheromone and oxygen avoidance) are exaggerated, and this heightened acuity is associated with exaggerated locomotion (both during lethargus and in adults) (Cheung et al., 2005; Choi et al., 2013; Gray et al., 2004; Macosko et al., 2009). Mutations that increase (e.g. *npr-1*) and decrease (e.g. *tax-4* CNG and *osm-9* TRPV) RMG circuit activity are associated with locomotion arousal and quiescence respectively (Choi et al., 2013; Coates and de Bono, 2002; de Bono et al., 2002; Macosko et al., 2009).

We previously showed that locomotion quiescence during lethargus is dramatically reduced in *npr-1* mutants and that this effect requires increased RMG sensory activity (Choi et al., 2013). Subsequent studies showed that in microfluidic chambers *npr-1* mutants have modest defects in lethargus quiescence when sensory cues are minimized but that dramatic quiescence defects are observed following brief stimulation with light or vibration (Nagy et al., 2014a; Nagy et al., 2014b). Taken together, these papers suggest that *npr-1* mutants exhibit aroused locomotion as a consequence of enhanced sensory activity.

The arousing effects of the RMG circuit are mediated in part by secretion of a neuropeptide, pigment dispersing factor (PDF-1) (Choi et al., 2013). Activation of PDF receptors (PDFR-1) in peripheral mechanosensory neurons enhances sensitivity to vibration, thereby accelerating locomotion. Thus, sensory evoked activity in the RMG circuit arouses locomotion during lethargus through changes in PDF-1 and PDFR-1

signaling. These results raise several interesting questions. Which specific sensory neurons are responsible for arousal? Does the RMG circuit regulate arousal via multiple outputs (i.e. in addition to PDF-1)? Does the RMG circuit function similarly during lethargus and in adults? Is diminished sensory acuity during lethargus required for behavioral quiescence?

Here we show that glutamatergic transmission promotes arousal, we identify glutamatergic neurons and glutamate receptors that mediate arousal, and we show that arousal occurs by distinct mechanisms in lethargus and adult animals.

Results

Cholinergic transmission at NMJs is increased in *npr-1* adults

Adult *npr-1* mutants exhibit accelerated locomotion (Fig. 2.1), as shown in prior studies (de Bono and Bargmann, 1998). Faster adult locomotion suggests that locomotion circuit activity has been altered. Consistent with this idea, *npr-1* mutant adults have enhanced sensitivity to the paralytic effects of a cholinesterase inhibitor (aldicarb) (Fig. 2.2) (Vashlishan et al., 2008), indicating increased excitatory transmission at neuromuscular junctions (NMJs).

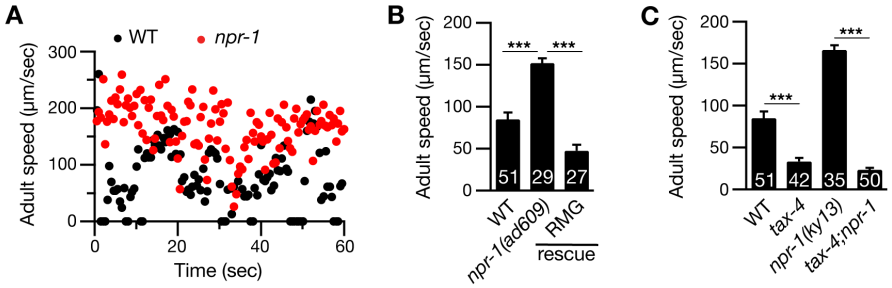

Figure 2.1 *npr-1* regulates adult locomotion. Locomotion behavior of single adult worms was analyzed for the indicated genotypes. Instantaneous locomotion velocity (A) and average locomotion velocity (B-C) are plotted. (A-C) The *npr-1* adult locomotion defect was rescued by transgenes expressing NPR-1 in the RMG circuit (RMG rescue, *flp-21* promoter), and suppressed in double mutants lacking TAX-4/CNG channels. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant).

To more directly assess changes in synaptic transmission, we recorded miniature excitatory post-synaptic currents (mEPSCs) in body muscles, which are evoked by acetylcholine (ACh) release at NMJs. The mEPSC rate observed in *npr-1* adults was significantly higher than in wild type controls while mEPSC amplitudes were unaltered (Fig. 2.3). Faster mEPSC rates suggest that ACh release from motor neurons was increased whereas unaltered mEPSC

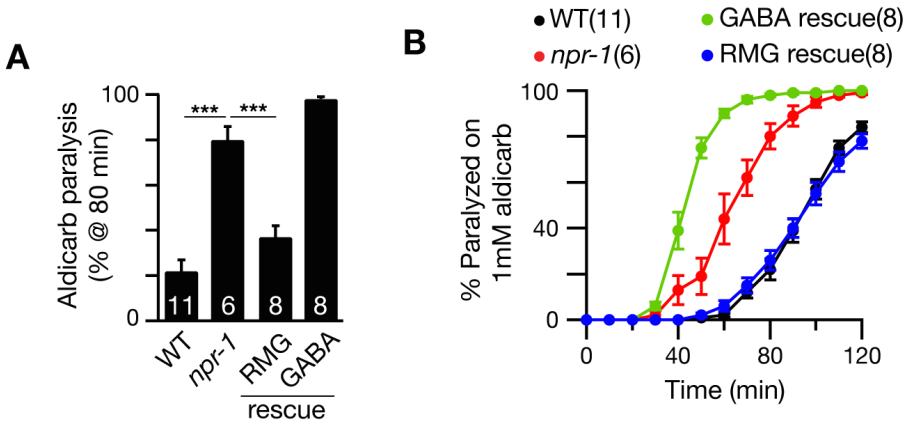

Figure 2.2 *npr-1* is hypersensitive to aldicarb-induced paralysis. The percentage of animals paralyzed on 1 mM aldicarb at 80 min were plotted for the indicated genotypes (A). The number of trials is indicated for each genotype. Full time courses (120 min) of aldicarb-induced paralysis are shown (B). The *npr-1* aldicarb hypersensitivity was rescued by transgenes expressing NPR-1 in the RMG circuit (RMG rescue, *flp-21* promoter) but not by those expressed in GABAergic neurons (GABA rescue, *unc-25* and *unc-30* promoters). Error bars indicate SEM. Values that differ significantly are indicated (*, p <0.05; **, p <0.01; ***, p <0.001; ns, not significant).

amplitudes imply that muscle responsiveness to secreted ACh was unaffected. By contrast, neither ACh release evoked by depolarizing motor neurons with a stimulating electrode (evoked EPSCs), nor transmission at GABAergic NMJs (assessed by miniature

inhibitory post-synaptic currents, mIPSCs) was altered in *npr-1* mutants (Fig. 2.4). This constellation of electrophysiological defects suggests that tonic ACh release (assessed by mEPSC rate) was enhanced in *npr-1* mutants, whereas other forms of neurotransmitter release (evoked ACh release and tonic GABA release) were unaffected. Enhanced tonic ACh release at NMJs could account for the accelerated locomotion rate observed in *npr-1* adults.

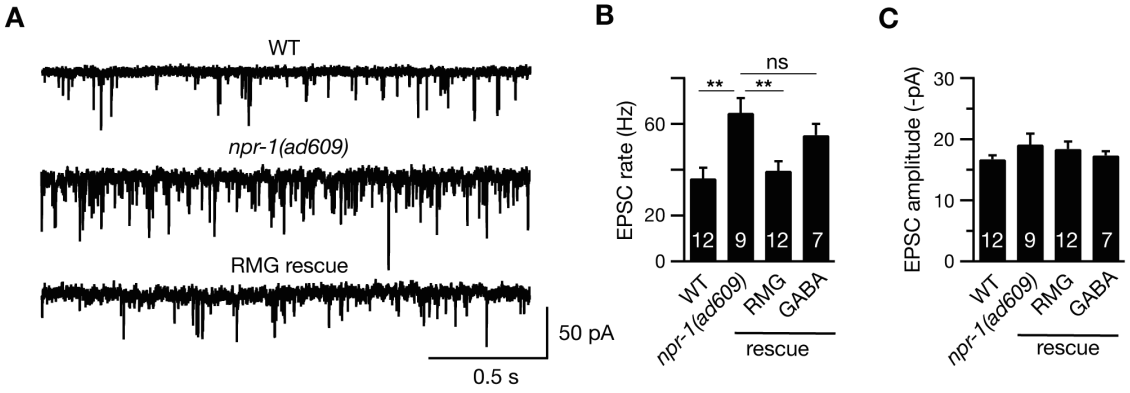

Figure 2.3 Cholinergic transmission at NMJs is enhanced by increased sensory activity in *npr-1* adults. mEPSCs were recorded from body wall muscles of adult worms for the indicated genotypes. Representative traces of mEPSCs (A) and summary data are shown (B-C). The *npr-1* cholinergic transmission defect was rescued by transgenes expressing NPR-1 in the RMG circuit (RMG rescue, *flp-21* promoter) but not by those expressed in GABAergic neurons (GABA rescue, *unc-30* promoter). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant).

Enhanced cholinergic transmission in *npr-1* adults is caused by increased sensory activity

Prior studies showed that several behavioral phenotypes exhibited by *npr-1* mutants are caused by enhanced sensitivity to environmental cues. In particular, sensory responses mediated by the RMG circuit are enhanced in *npr-1* mutants (Coates and de Bono, 2002; de Bono et al., 2002; Macosko et al., 2009) and this enhanced sensory acuity is required for accelerated locomotion rates during lethargus (Choi et al., 2013; Nagy et al., 2014b). We did several experiments to determine if enhanced RMG circuit activity is also required for increased cholinergic transmission in *npr-1* adults. A transgene restoring *npr-1* expression in the RMG circuit (using the *flp-21* promoter) rescued the accelerated locomotion (Fig. 2.1B), enhanced aldicarb sensitivity (Fig. 2.2), and faster mEPSC rate (Fig. 2.3) defects of *npr-1* adults. By contrast, an *npr-1* transgene expressed in GABAergic neurons lacked rescuing activity (Fig. 2.2 and 2.3). These results indicate that NPR-1 acts in the RMG circuit to slow adult locomotion. Similarly, mutations inactivating ion channels required for sensory transduction (TAX-4/CNG and OCR-2/TRPV) in the RMG circuit suppressed the *npr-1* adult locomotion (Fig. 2.1C), aldicarb sensitivity (Fig. 2.5A-D), and mEPSC rate (Fig. 2.5E-G) defects. Collectively, these results suggest that the accelerated adult locomotion exhibited by *npr-1* mutants is caused by heightened activity in the RMG sensory circuit and, consequently, corresponds to an aroused state.

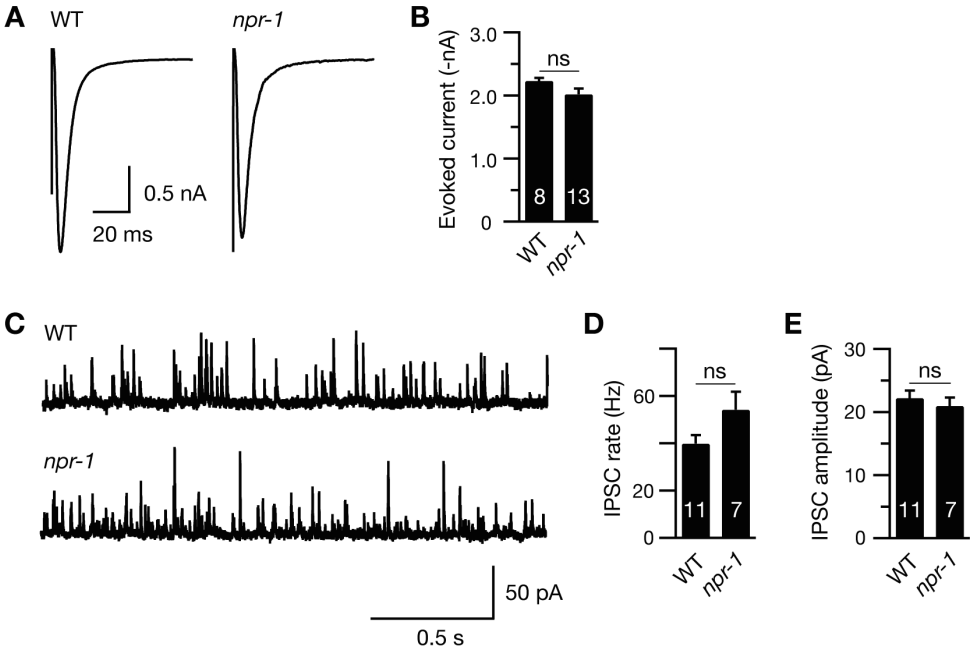

Figure 2.4 Stimulus-evoked EPSCs and endogenous IPSCs are normal in *npr-1* adults. Stimulus-evoked EPSCs (A-B) and mIPSCs (C-E) were recorded from body wall muscles of adult worms for the indicated genotypes. Averaged traces of stimulus-evoked EPSCs (A), representative traces of mIPSCs (C), and summary data are shown (B, D, and E). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM (ns, not significant).

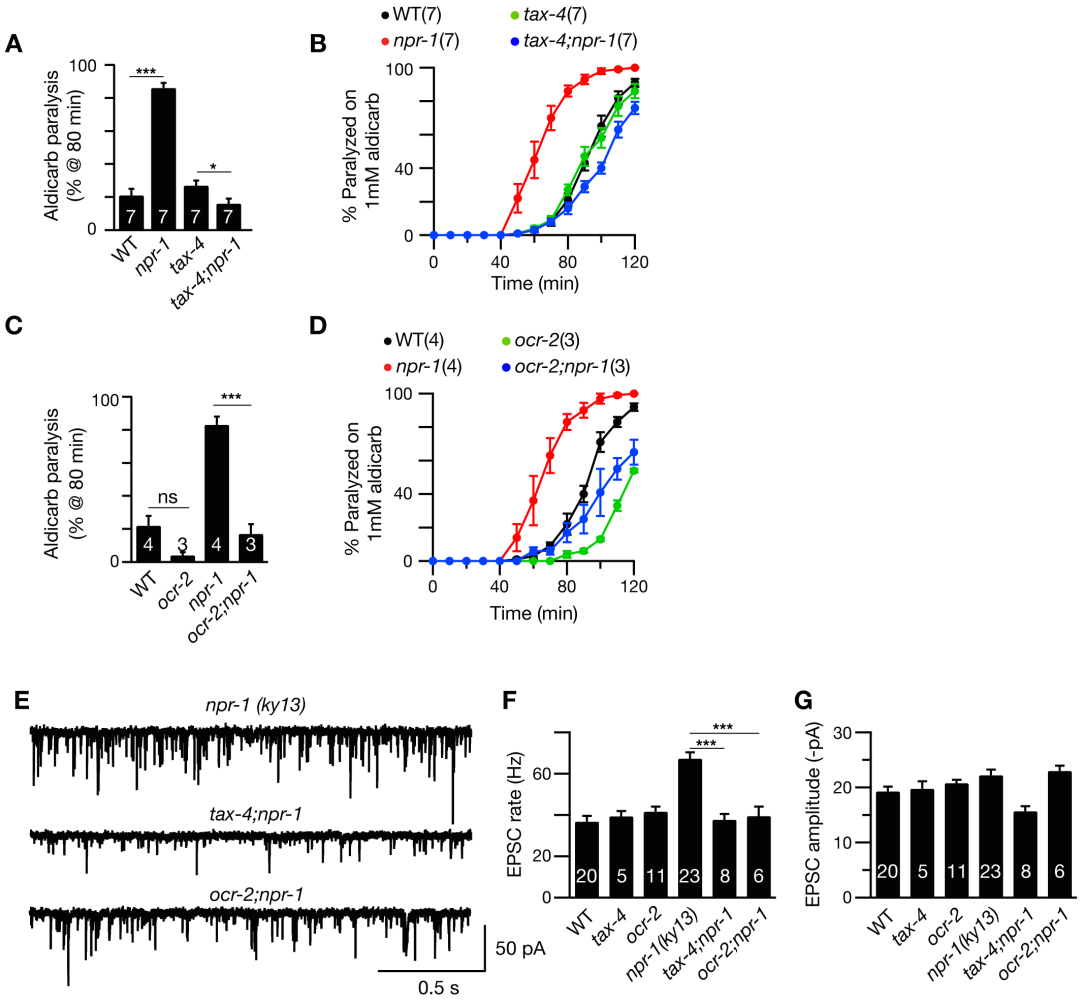

Figure 2.5 *npr-1* aldicarb and transmission defects require sensory transduction. The percentage of animals paralyzed on 1 mM aldicarb at 80 min were plotted for the indicated genotypes (A,C). The number of trials is indicated for each genotype. Full time courses (120 min) of aldicarb-induced paralysis are shown (B,D). The *npr-1* aldicarb hypersensitivity was blocked by mutations inactivating TAX-4/CNG channels or OCR-2/TRPV channels (A-D). mEPSCs were recorded from body wall muscles of adult worms for the indicated genotypes. Representative traces of mEPSCs (E) and summary data are shown (F-G). The *npr-1* cholinergic transmission defect was abolished by mutations inactivating TAX-4 or OCR-2. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, p <0.05; **, p <0.01; ***, p <0.001; ns, not significant).

Inactivating PDF signaling does not prevent aroused locomotion in *npr-1* adults

We previously showed that the lethargus quiescence defects exhibited by *npr-1* mutants are caused by increased secretion of Pigment dispersing factor (PDF-1) by cells in the RMG circuit (Choi et al., 2013). Because PDF-1 secretion is also increased in *npr-1* adults (Choi et al., 2013), we tested the idea that the hyperactive adult locomotion of *npr-1* mutants is also caused by increased PDF signaling. Contrary to this idea, we found that *pdf-1* and *pdfr-1* (PDF Receptor-1) mutations reduced but did not eliminate the aldicarb hypersensitivity, accelerated locomotion, and increased mEPSC rate (Fig. 2.6) defects of *npr-1* adults. Collectively, these results suggest that additional excitatory outputs from the RMG circuit (i.e. beyond PDF-1) must contribute to the aroused locomotion of *npr-1* adults.

Glutamate released by sensory neurons is required for *npr-1* locomotion and EPSC defects

Many *C. elegans* sensory neurons are glutamatergic, including two neurons in the RMG circuit (ASH and ASK) and the body touch neurons (Lee et al., 1999). To determine if glutamate release by sensory neurons is required for accelerated locomotion in *npr-1* mutants, we analyzed mutations that inactivate the vesicular glutamate transporter (*eat-4* VGLUT), which is primarily expressed in sensory neurons (Lee et al., 1999). *eat-4* VGLUT mutations blocked the increased motile fraction and locomotion speed of *npr-1* mutants both during the L4-Adult (L4/A) molt and in adults (Fig. 2.7). *eat-4* mutations also blocked the hypersensitivity to aldicarb (Fig. 2.8) and increased mEPSC rate (Fig. 2.9) defects of *npr-1* adults.

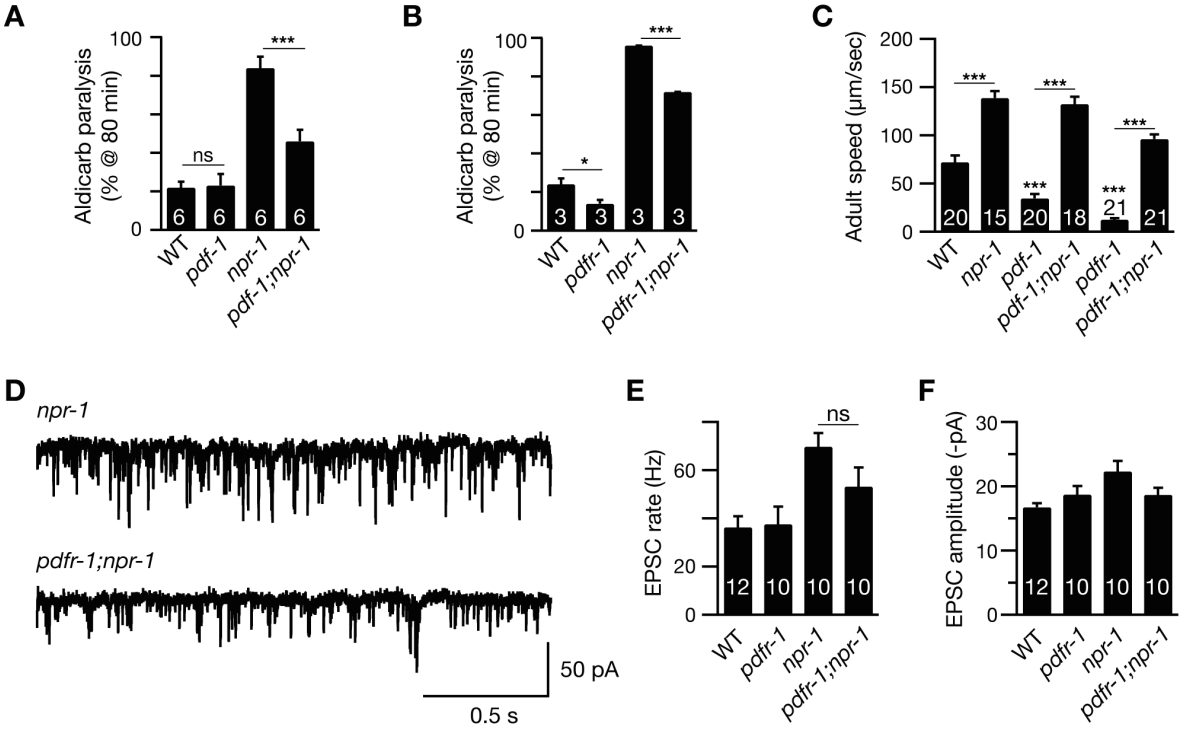

Figure 2.6 Inactivating PDF signaling does not prevent aroused locomotion in *npr-1* adults. (A-B) The *npr-1* aldicarb hypersensitivity was decreased but not abolished by mutations inactivating PDF-1 or PDFR-1. The percentage of animals paralyzed on 1 mM aldicarb at 80 min were plotted for the indicated genotypes. The number of trials is indicated for each genotype. (C) Locomotion behavior of single adult worms was analyzed for the indicated genotypes. The *npr-1* adult locomotion defect was not blocked by mutations inactivating PDF-1 or PDFR-1. (D-F) mEPSCs were recorded from body wall muscles of adult worms for the indicated genotypes. Representative traces of mEPSCs (D) and summary data are shown (E-F). The *npr-1* cholinergic transmission defect was not suppressed by mutations inactivating PDFR-1. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*,p<0.05;**,p<0.01;***, p <0.001; ns, not significant)

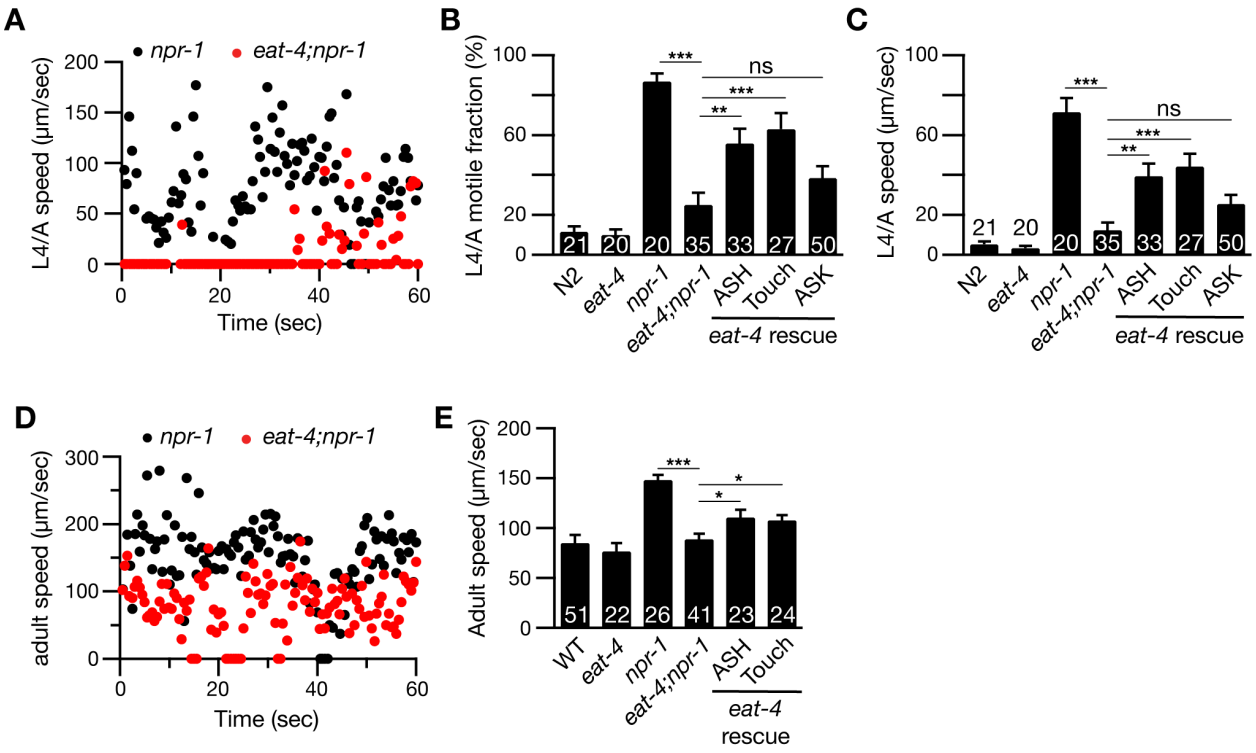

Figure 2.7 Glutamate released by sensory neurons is required for the *npr-1* locomotion defects. Locomotion behavior of single worms during the L4/A lethargus (A-C) and in adults (D-E) was analyzed in the indicated genotypes. Instantaneous locomotion velocity (A, D), average motile fraction (B), and average locomotion velocity (C, E) are plotted. The *npr-1* locomotion defect was suppressed by mutations inactivating EAT-4/VGLUT, and partially reinstated by transgenes expressing EAT-4 in ASH neurons (*sra-6* promoter) and touch neurons (*mec-4* promoter) in *eat-4;npr-1* double mutants using the indicated promoters. An EAT-4 transgene expressed in ASK neurons (*sra-9* promoter) lacked rescuing activity. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, p <0.05; **, p <0.01; ***, p <0.001; ns, not significant).

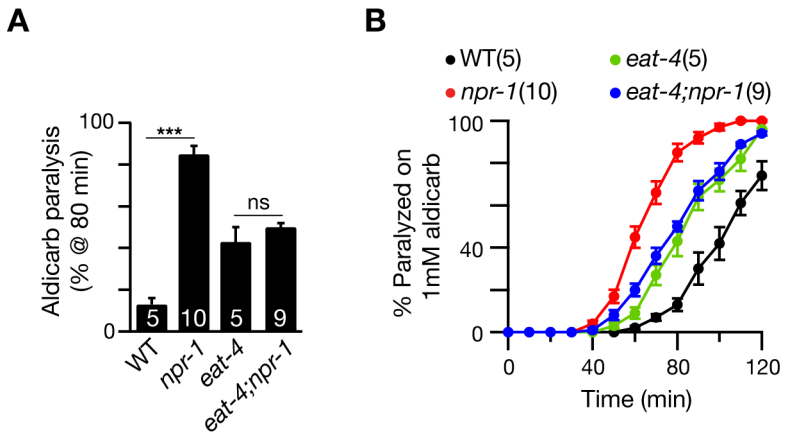

Figure 2.8 Glutamate release is required for the *npr-1* aldicarb hypersensitivity. The *npr-1* aldicarb hypersensitivity was suppressed by mutations inactivating EAT-4/VGLUT. The percentage of animals paralyzed on 1 mM aldicarb at 80 min were plotted for the indicated genotypes (A). The number of trials is indicated for each genotype. Full time courses of aldicarb-induced paralysis are shown (B). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, p <0.05; **, p <0.01; ***, p <0.001; ns, not significant).

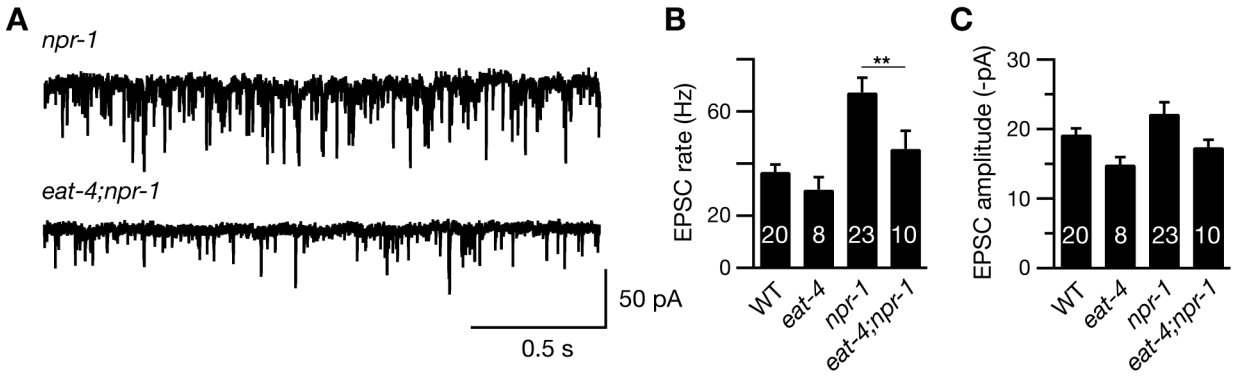

Figure 2.9 Glutamate release is required for the *npr-1* cholinergic transmission defects. The *npr-1* cholinergic transmission defect was abolished by mutations inactivating EAT-4/VGLUT. mEPSCs were recorded from body wall muscles of adult worms for the indicated genotypes. Representative traces of mEPSCs (A) and summary data are shown (B-C). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, p <0.05; **, p <0.01; ***, p <0.001; ns, not significant).

Transgenes restoring EAT-4 expression in touch neurons and ASH neurons partially reinstated both lethargus and adult locomotion defects in *eat-4*; *npr-1* double mutants, whereas transgenes expressed in ASK lacked rescuing activity (Fig. 2.7). *eat-4* transgenes had no effect on lethargus quiescence in wild type animals (Fig. 2.10). These results suggest that glutamate released by ASH and touch neurons arouses locomotion in L4/A and adult *npr-1* mutants.

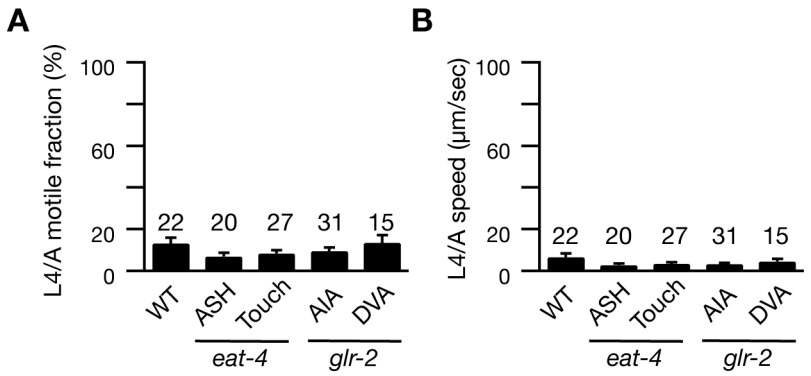

Figure 2.10 Transgenic expression of EAT-4 or GLR-2 in WT worms has no effect on lethargus quiescence. Locomotion behavior of single worms during the L4/A lethargus (A-B) was analyzed in the indicated genotypes. Average motile fraction (A), and average locomotion velocity (B) are plotted. Transgenes that re-instated lethargus quiescence defects in *eat-4;npr-1* (*sra-6* or *mec-4* promoted EAT-4, Fig. 2.7) or *glr-2;npr-1* (*gcy-28d* or *nlp-12* promoted GLR-2, Fig. 2.14) double mutants had no effect on lethargus quiescence in wild type worms. The number of animals analyzed is indicated for each genotype.

ASH activity is associated with locomotion arousal

The preceding results suggest that ASH synaptic output arouses locomotion in *npr-1* mutants. We did several additional experiments to test this idea. If altered ASH

output were required for aroused locomotion, we would expect that *npr-1* mutants lacking ASH neurons would have increased locomotion quiescence. To test this idea, we induced ASH cell death with a transgene that expresses the pro-apoptotic caspase CED-3. Killing ASH significantly decreased the L4/A motile fraction and locomotion rate in *npr-1* mutants (Fig. 2.11). By contrast, ASH ablation had little effect on the locomotion rate of *npr-1* adults (Fig. 2.11).

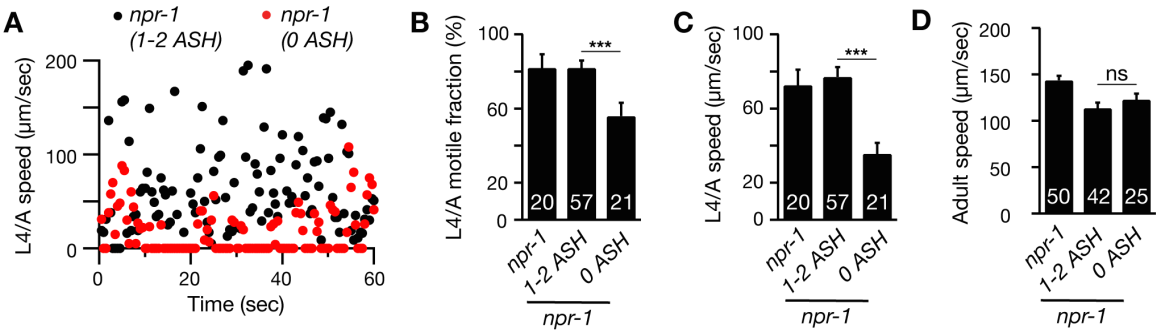

Figure 2.11 ASH neurons are required for the *npr-1* locomotion quiescence defect. Locomotion behavior during the L4/A lethargus (A-C) and in adults (D) of single worms whose ASH neurons were ablated by transgenic overexpression of CED-3 in ASH neurons (*sra-6* promoter) was analyzed in the indicated genotypes. Animals were analyzed by fluorescence microscopy after locomotion recordings to determine if ASH neurons were ablated (1-2 ASH: animals with 1 or 2 ASH intact neurons; 0 ASH: animals lacking viable ASH neurons). Instantaneous locomotion velocity (A), average motile fraction (B), and average locomotion velocity (C-D) are plotted. The *npr-1* locomotion defect during the L4/A lethargus, but not in adults, was partially suppressed in the transgenic animals in which both of ASH neurons were ablated (0 ASH). The number of trials is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, p <0.001; ns, not significant).

To determine if ASH activity is increased in *npr-1* mutants during lethargus, we examined sensory-evoked calcium responses in ASH, using the genetically encoded calcium indicator Cameleon. ASH mediates avoidance responses to copper and hyper-osmotic stimuli. Consistent with a recent study (Cho and Sternberg, 2014), the magnitude of copper and glycerol-evoked calcium transients in ASH was significantly decreased during lethargus in wild-type animals (Fig. 2.12). Decreased ASH responsiveness to copper and glycerol during L4/A lethargus was blocked in *npr-1* mutants, whereas ASH responsiveness in adults was unaltered in *npr-1* mutants (Fig. 2.12). Transgenes expressing NPR-1 in the RMG circuit (using the *flp-21* promoter) or in ASH (using the *sra-6* promoter) reinstated the L4/A decrease in copper and glycerol-evoked ASH calcium transients in *npr-1* mutants (Fig. 2.12 C-D, G-H). These results suggest that NPR-1 acts in ASH to inhibit sensory responses and that increased ASH activity is required for accelerated locomotion of *npr-1* mutants during lethargus but not in adults.

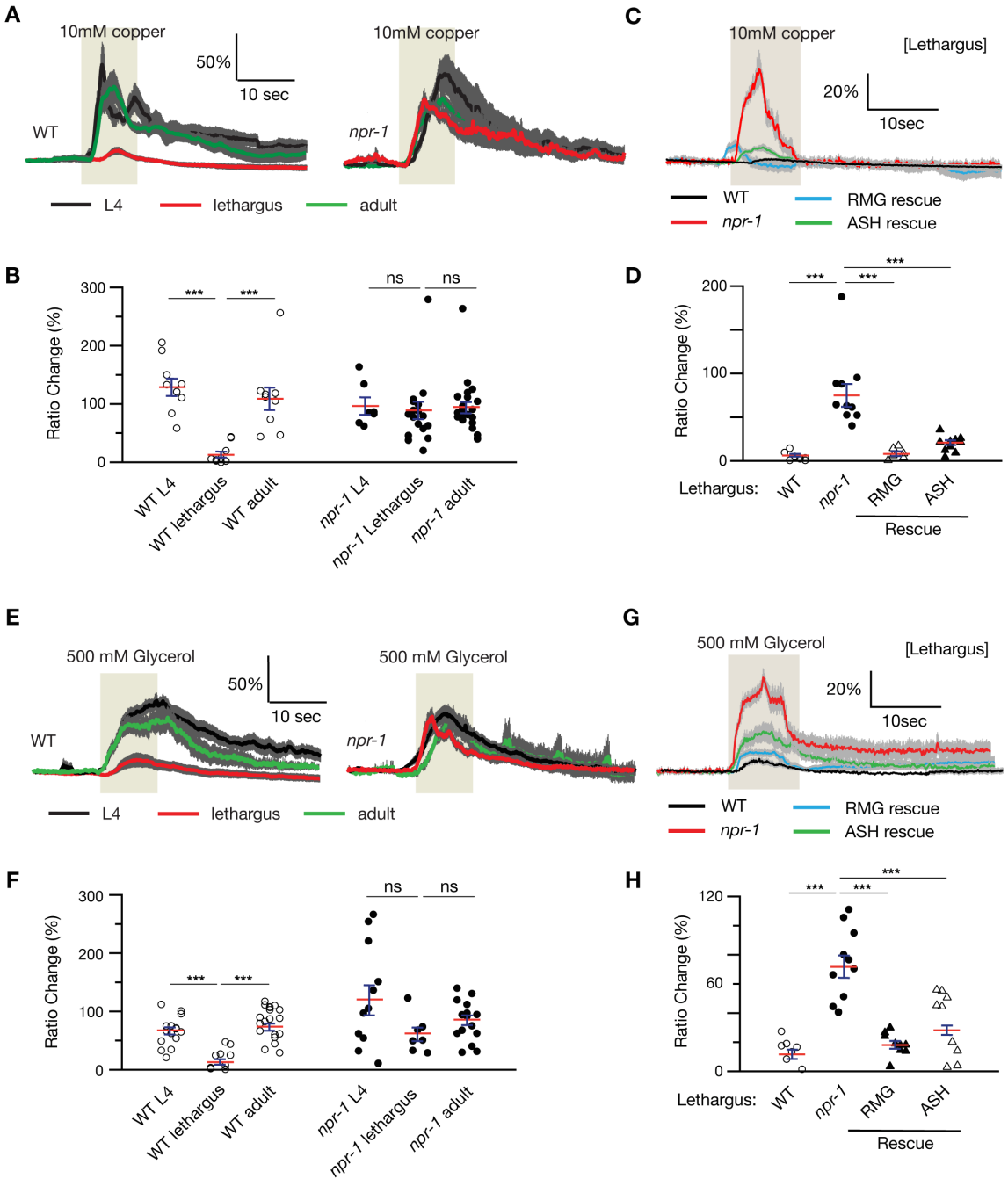

Figure 2.12 NPR-1 is required for the decreased copper- and glycerol-evoked calcium transients in ASH during L4/A lethargus. Copper-evoked (A-D) and glycerol-evoked (E-H) calcium transients in ASH were analyzed in L4, L4/A, and adults of the indicated genotypes using cameleon as a calcium indicator. Averaged responses (A,C,E, G), and the amplitudes of

Figure 2.12 (Continued). individual trials (B,D,F, H) are shown for each genotype. Each trace represents the average percentage change in YFP/CFP fluorescence ratio. The light tan rectangle indicates the duration for which 10 mM copper or 500 mM glycerol was applied. Dark gray shading of each trace indicates SEM of the mean response. (A-B,E-F) Copper-evoked and glycerol-evoked calcium transients in ASH neurons were significantly reduced during L4/A lethargus, and this effect was abolished in *npr-1* mutants. (C-D,G-H) This defect during L4/A lethargus was rescued by transgenes expressing NPR-1 in the RMG circuit (RMG rescue, *flp-21 promoter*) or in ASH neurons (ASH rescue*, sra-6 promoter*). Values that differ significantly are indicated (***, $p < 0.001$; ns, not significant).

To determine if increased ASH activity is sufficient to arouse locomotion, we analyzed locomotion after artificially depolarizing ASH neurons. For this experiment, we utilized transgenic animals that express rat TRPV1 capsaicin receptors in ASH neurons (Tobin et al., 2002). In these animals, capsaicin treatment evokes ASH-mediated avoidance behaviors (Tobin et al., 2002). A 5-hour capsaicin treatment had little effect on L4/A motile fraction and locomotion velocity (Choi et al., 2013), whereas capsaicin treatment significantly accelerated adult locomotion (Fig. 2.13A) and increased aldicarb sensitivity (Fig. 2.13B). These effects were not observed in animals lacking TRPV1 expression in ASH neurons (Fig. 2.13A). Thus, forced ASH depolarization was sufficient to arouse adult but not lethargus locomotion. Collectively, these results suggest that diminished and heightened ASH activity is associated with locomotion quiescence and arousal respectively; however, the magnitude of ASH's arousing effects differ between lethargus and adult animals.

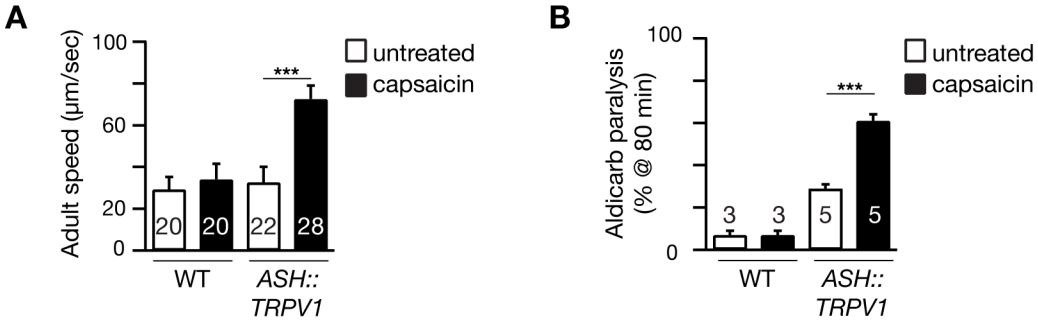

Figure 2.13 Forced depolarization of ASH neurons aroused adult locomotion and increased aldicarb sensitivity. Locomotion behavior of adult transgenic worms was analyzed with or without capsaicin treatment (5 hours). Average locomotion velocity (I) is plotted. Capsaicin treatment increased adult locomotion velocity in transgenic animals expressing TRPV1 in ASH neurons, but not in wild type controls. The number of animals analyzed is indicated for each genotype. (J) The percentage of animals paralyzed on 1 mM aldicarb at 80 min with or without capsaicin treatment (2-3 hours pretreatment) were plotted for the indicated genotypes. The number of trials is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, p <0.001; ns, not significant).

GLR-2 AMPA receptors are required for the *npr-1* lethargus defect

Which glutamate receptors arouse locomotion in *npr-1* mutants? Glutamate-activated cation channels, AMPA (GLR-1 and -2) and NMDA (NMR-1 and -2) receptors, mediate excitatory transmission at ASH-interneuron (Brockie et al., 2001; Hart et al., 1995; Maricq et al., 1995). The *npr-1* L4/A quiescence defect was abolished in *glr-2; npr-1* double mutants (Fig. 2.14 A-C), while *glr-1* mutations had no effect (Fig. 2.14 D-E).

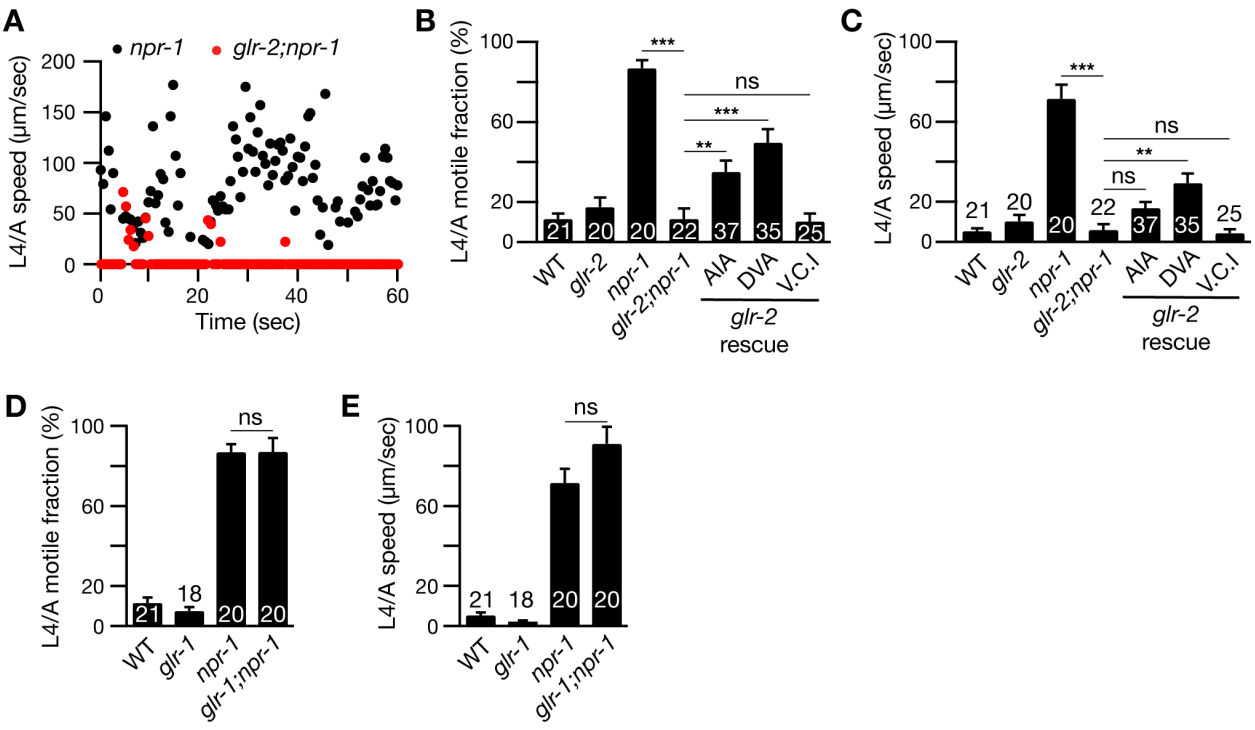

Figure 2.14 GLR-2 AMPA receptors are required for the *npr-1* lethargus defect. Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Instantaneous locomotion velocity (A), average motile fraction (B, D), and average locomotion velocity (C, E) are plotted. (A-C) The *npr-1* locomotion defect during L4/A lethargus was suppressed by mutations inactivating *glr-2* AMPA receptors, and partially reinstated by transgenes expressing GLR-2 in AIA (*gcy-28(d)* promoter) and DVA (*nlp-12* promoter) neurons, but not in Ventral Cord Interneurons (V.C.I., *glr-1* promoter) in *glr-2;npr-1* double mutants using the indicated promoters. (D-E) *glr-1* mutations had no suppressing effect. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (**, p <0.01; ***, p <0.001; ns, not significant).

By contrast, *glr-1, glr-2,* and *nmr-1* mutations had little effect on *npr-1* adult locomotion (Fig. 2.15 A-B). Similarly, *glr-2* mutations did not block the increased

mEPSC rate in *npr-1* adults (Fig. 2.15 C). These results suggest that GLR-2 AMPA receptors are specifically required for the aroused locomotion during the L4/A lethargus in *npr-1* mutants.

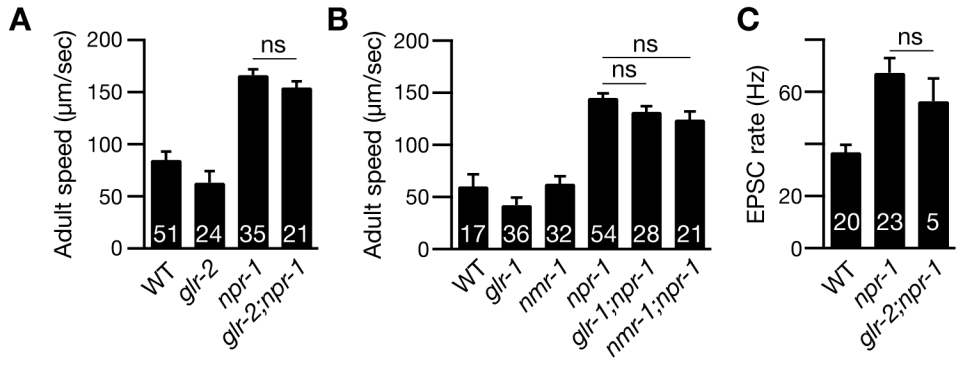

Figure 2.15 GLR-2, GLR-1 and NMR-1 glutamate receptors are not required for the increased locomotion in *npr-1* adults. Locomotion behavior of single adult worms was analyzed in the indicated genotypes (A-B). Average locomotion velocity is plotted. The locomotion defect in *npr-1* adults was not suppressed by mutations inactivating *glr-2* (A)*, glr-1* or *nmr-1* glutamate receptors (B). mEPSCs were recorded from body wall muscles of the adult worms for the indicated genotypes. Summary data are shown. *glr-2* mutations did not block the increased mEPSC rate in *npr-1* adults (C). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (ns, not significant).

GLR-2 AMPA receptors act in AIA and DVA to mediate arousal

Which synaptic targets of ASH and touch neurons mediate locomotion arousal? To address this question, we identified the neurons in which GLR-2 function is required. Aroused L4/A locomotion requires GLR-2 but not GLR-1 receptors; consequently, we reasoned that the relevant neurons are likely to express GLR-2 but not GLR-1. GLR-1

and GLR-2 are co-expressed in many neurons; however, a few GLR-2-expressing neurons lack GLR-1, including DVA (a stretch-activated neuron) and AIA (an interneuron in the head ganglia) (Brockie et al., 2001; Hart et al., 1995; Maricq et al., 1995). The L4/A quiescence defect was partially restored in *glr-2; npr-1* double mutants by transgenes expressing GLR-2 in DVA and AIA neurons, whereas transgenes expressed in the ventral cord interneurons (using the *glr-1* promoter) failed to rescue (Fig. 2.14). Transgenic expression of GLR-2 in DVA or AIA had no effect on lethargus quiescence in wild type worms (Fig. 2.10). These results suggest that GLR-2 AMPA receptors expressed in AIA and DVA neurons arouse L4/A locomotion in *npr-1* mutants. DVA receives direct synaptic input from the touch neuron PLM while AIA receives direct input from ASH (White et al., 1986a). Thus, increased transmission at ASH-AIA and PLM-DVA synapses could account for GLR-2's effects on locomotion rate. Because we only observed partial rescue by *glr-2* transgenes expressed in AIA and DVA, it is likely the GLR-2 function is required in additional (as yet unidentified) neurons.

How do AIA and DVA arouse locomotion? AIA neurons provide synaptic input to ASK and ASI, both of which express PDF-1 (Choi et al., 2013; Janssen et al., 2009). Thus, heightened AIA activity could arouse locomotion by enhancing PDF-1 secretion. To assess the level of PDF-1 secretion, we analyzed PDF-1::YFP fluorescence in the endolysosomal compartment of coelomocytes, which are specialized scavenger cells that internalize proteins secreted into the body cavity (Fares and Greenwald, 2001; Sieburth et al., 2007). Inactivating GLR-2 did not alter PDF-1::YFP fluorescence in coelomocytes in both adult and L4/A animals (Fig. 2.16). These results suggest that the arousing effects of

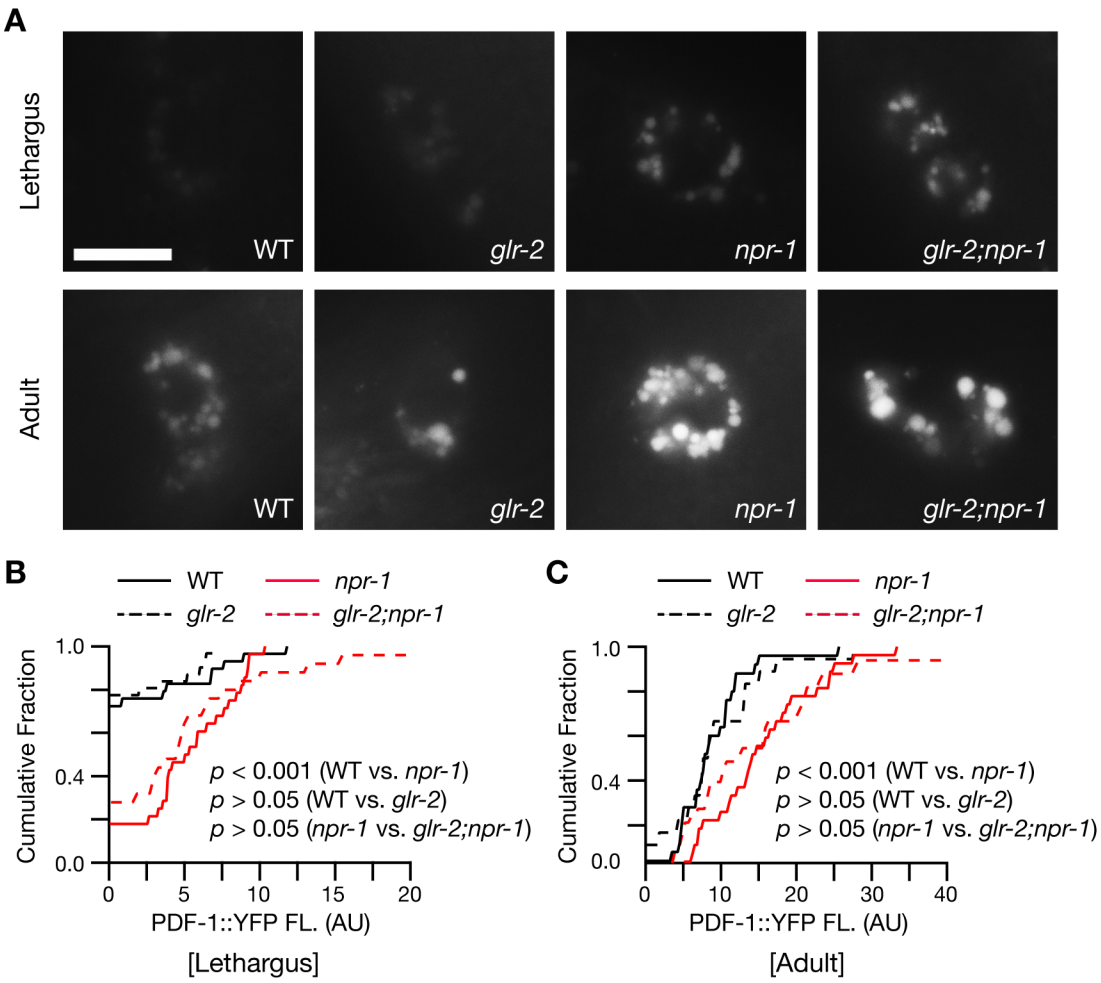

Figure 2.16 PDF-1 secretion is not altered in *glr-2* mutants. PDF-1 secretion was analyzed in the indicated genotypes. YFP-tagged PDF-1 was expressed with the *pdf-1* promoter. Representative images (A) and summary data (cumulative fraction) (B-C) are shown for coelomocyte fluorescence in L4/A lethargus and 1-day old adults of the indicated genotypes. PDF-1::YFP coelomocyte fluorescence was dramatically increased in *npr-1* mutants during the L4/A lethargus and in adults as previously reported (Choi et al., 2013). Mutations inactivating GLR-2 did not alter PDF-1::YFP coelomocyte fluorescence during L4/A lethargus (B) and in adults (C) in either wild type or *npr-1* mutants. Scale bar indicates 10 μm. *p* values are indicated for each comparison (Kolmogorov-Smirnov test).

GLR-2 are not mediated by changes in PDF secretion. DVA neurons receive direct synaptic input from the PLM touch neurons (White et al., 1986b), and secrete NLP-12 (a neuropeptide that accelerates locomotion) (Hu et al., 2011). Thus, increased DVA activity could contribute to locomotion arousal in *npr-1* mutants. Three results support this idea. First, PLM neurons exhibit enhanced touch-evoked calcium responses in adult *npr-1* mutants (Fig. 2.17). Thus, PLM neurons have increased sensory acuity in *npr-1* mutants, similar to the effect we previously showed for ALM neurons (Choi et al., 2013). Second, inducing DVA cell death (with a CED-3 transgene) significantly reduced *npr-1* locomotion rate during L4/A lethargus, but not in adults (Fig. 2.18). Third, DVA secretion of NLP-12 is significantly increased in *npr-1* mutants (Hu et al., 2011), indicating increased DVA activity. These results suggest that PLM neurons provide enhanced excitatory input to DVA in *npr-1* mutants, which promotes aroused L4/A locomotion.

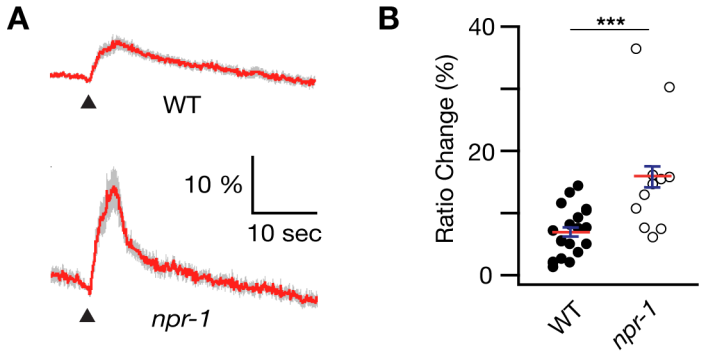

Figure 2.17 PLM touch sensitivity is increased in *npr-1* mutants. Touch-evoked calcium transients in PLM were analyzed using cameleon as a calcium indicator. Responses were analyzed in adult animals. Averaged responses (A) and the amplitudes of individual trials (B) are shown for each genotype. Each red trace represents the average percentage change in YFP/CFP fluorescence

Figure 2.17 (Continued). ratio. The black triangle indicates the time at which the mechanical stimulus was applied. Gray shading indicates the response SEM. Touch-evoked calcium transients in adult PLM neurons were significantly larger in *npr-1* mutants.

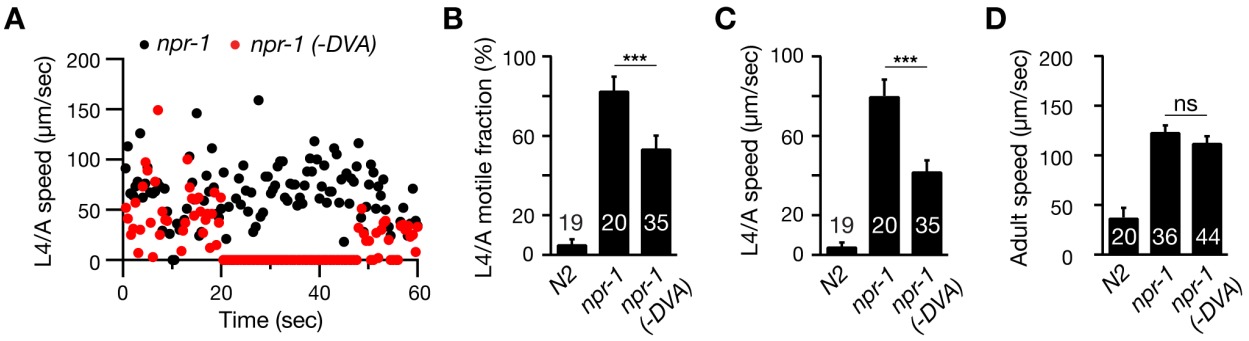

Figure 2.18 DVA is required for the *npr-1* locomotion quiescence defect. Locomotion behavior during the L4/A lethargus (A-C) and in adults (D) of single worms whose DVA neuron is ablated by transgenic overexpression of CED-3 in DVA neuron (*nlp-12* promoter) was analyzed in the indicated genotypes. Animals were analyzed by fluorescence microscopy after locomotion recordings to determine if DVA was ablated. The *npr-1* locomotion defect during the L4/A lethargus, but not in adults, was partially suppressed in the transgenic animals in which DVA was ablated (-DVA). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (**, p <0.01; ***, p <0.001; ns, not significant).

Discussion

To investigate the circuit mechanisms for arousal, we analyzed the locomotion of *npr-1* mutants in awake (adult) and quiescent (lethargus) states. Our results lead to five conclusions. First, multiple classes of sensory neurons contribute to arousal. Second, diminished sensory acuity is a circuit mechanism for promoting behavioral quiescence. Third, glutamate and neuropeptides are utilized as excitatory outputs from sensory neurons to arouse locomotion. Fourth, different mechanisms are utilized to arouse locomotion at different times during development. And fifth, we provide further evidence that arousal mechanisms are conserved across phylogeny.

A broad network of sensory neurons contribute to arousal

Multiple classes of sensory neurons arouse locomotion during lethargus and in adults, including: mechanosensory neurons (ALM and PLM), a nociceptive neuron (ASH), a pheromone sensing neuron (ASK), and a stretch sensing neuron (DVA). Lethargus quiescence is accompanied by diminished sensory-evoked responses in ALM, PLM, and ASH (this study and (Cho and Sternberg, 2014; Choi et al., 2013; Schwarz et al., 2011)). PDF-1 secretion from ASK neurons is significantly reduced during lethargus, implying that ASK neurons also have diminished activity during lethargus (Choi et al., 2013). *npr-1* mutations prevent the dampened ALM (mechanosensory) and ASH (nociceptive) responses during lethargus and this was accompanied by decreased locomotion quiescence (this study and (Choi et al., 2013)). The arousing effects of *npr-1* mutations are blocked (or diminished) by mutations that decrease sensory responsiveness (e.g. *tax-4* CNG and *osm-9* TRPV mutations) (Choi et al., 2013), or by ablating sensory

neurons (e.g. ASH and DVA). Forced activation of ASH neurons arouses adult locomotion. Collectively, these results imply that a broad network of sensory neurons arouses locomotion, which allows *C. elegans* to adapt its behavior across a broad range of developmental and physiological circumstances.

Sensory gating control as a mechanism for producing quiescence and arousal

NPR-1 promotes behavioral quiescence by diminishing the sensitivity of many sensory modalities. NPR-1 directly inhibits ASH responses and indirectly inhibits other sensory neurons (ALM, PLM, and DVA) via decreased glutamate and neuropeptide release. Thus, gating of sensory perception by NPR-1 provides a circuit mechanism for producing aroused and quiescent locomotion in *C. elegans*.

Our results do not exclude the possibility that additional mechanisms (beyond sensory gating by NPR-1) contribute to arousal and quiescence. Both quiescence (during lethargus) and arousal (following molts) persist in microfluidic chambers where many sensory cues are minimized (Nagy et al., 2014a). In particular, oxygen tension is likely to be very low in these chambers, which would greatly diminish NPR-1's effects on behavior (Cheung et al., 2005; Gray et al., 2004). Thus, the quiescence and arousal exhibited in microfluidic chambers implies that additional mechanisms beyond NPR-1 must contribute to expressing these behavioral states. It will be interesting to determine if these NPR-1 independent mechanisms also act by gating sensory activity.

Sensory-evoked glutamate and neuropeptide release arouses locomotion

Sensory neurons release glutamate and/or neuropeptides in response to external cues, which then engage downstream motor circuits in behavioral outputs. Our prior study shows that sensory-evoked PDF-1 secretion promotes locomotion arousal by enhancing touch neuron responsiveness. Neuropeptides also mediate arousal in flies (PDF) (Parisky et al., 2008), fish and mammals (orexin/hypocretin) (Prober et al., 2006; Sutcliffe and de Lecea, 2002).

Here we show that sensory evoked glutamate release also plays a role in arousal. Mutations inactivating the EAT-4/VGLUT decreased locomotion arousal in lethargus and in adults. EAT-4 is almost exclusively expressed in sensory neurons (Lee et al., 1999) and transgenes restoring EAT-4 expression in touch neurons and ASH neurons re-instates locomotion arousal in *npr-1* mutants. These results suggest that sensory neurons utilize both glutamate and neuropeptides as excitatory outputs to arouse locomotion.

Our results suggest that exaggerated glutamate release at ASH-AIA and PLM-DVA synapses arouses locomotion during lethargus in *npr-1* mutants. ASH and PLM neurons have enhanced sensory evoked activity in *npr-1* mutants, which is expected to produce enhanced glutamate release at ASH-AIA and PLM-DVA synapses. GLR-2 receptors are expressed in AIA and DVA. *glr-2* mutations block the aroused L4/A locomotion of *npr-1* mutants and arousal is re-instated by transgenes expressing GLR-2 in AIA and DVA. Finally, calcium responses in AIA (Macosko et al., 2009), and neuropeptide secretion from DVA (Hu et al., 2011) are both enhanced in *npr-1* mutants, indicating that these neurons have increased activity. We observed only partial rescue of aroused locomotion by transgenes restoring EAT-4 expression in ASH and touch neurons

or by those expressing GLR-2 in AIA or DVA; consequently, it is likely that glutamate released by other sensory neurons also contributes to the aroused L4/A locomotion in *npr-1* mutants.

Much less is known about the role of glutamate in arousal in other systems. Glutamate release has widespread effects throughout the brain in mammals, which complicates the analysis of its effects on arousal. Microinjection of glutamate or AMPA into lateral hypothalamic area increased locomotor activity and duration of waking episodes in rodents (Alam and Mallick, 2008; Li et al., 2011), while microdialysis of CNQX, an AMPA receptor antagonist, into the thalamus promotes sleep in cats (Juhasz et al., 1990). Glutamate also induces fictive locomotion in lamprey (Brodin et al., 1985). In these cases, however, the circuit mechanisms underlying glutamate's arousing effects are not known.

Comparing lethargus and adult arousal mechanisms

Mutants lacking NPR-1 exhibit accelerated locomotion in adults and during lethargus (Choi et al., 2013; de Bono et al., 2002). Several results suggest that locomotion arousal in adult and lethargus is established by a shared central sensory circuit. First, in both adult and lethargus, enhanced activity in the RMG sensory circuit accelerates locomotion, whereas decreased sensory transduction in the RMG circuit (i.e. by inactivating TAX-4 or OSM-9) abolishes *npr-1's* hyperactive locomotion defect (Choi et al., 2013; Macosko et al., 2009), suggesting that the RMG circuit activity stimulates arousal in both awake and quiescent states. Second, EAT-4 acts in ASH and touch neurons to mediate hyperactive locomotion of *npr-1* adult and lethargus stage animals,

suggesting that glutamate release from these sensory neurons is required for locomotion arousal in *npr-1* mutants.

On the other hand, several results suggest that the mechanisms that arouse locomotion differ between adult and lethargus animals. Inactivating GLR-2 AMPA receptors blocks the hyperactive locomotion of *npr-1* mutants during lethargus but not in adults. Aroused locomotion in *npr-1* adults persists in *glr-1, glr-2,* and *nmr-1* mutants, indicating that other glutamate receptors are responsible for arousing adult locomotion. Similarly, artificial activation of ASH accelerates adult but not lethargus locomotion. Collectively, our results suggest that multiple sensory circuits govern locomotion arousal throughout development but that the relative contribution of each circuit to arousal differs depending on the developmental stage.

Materials and Methods

Strains

Strain maintenance and genetic manipulation were performed as described (Brenner, 1974). Animals were cultivated at 20°C on agar nematode growth media (NGM) seeded with OP50 (for imaging and behavior) or HB101 *E.coli* (for electrophysiology). Wild type reference strain was N2 Bristol. Strains used in this study are as follows:

Mutant strains and integrants

KP6048 *npr-1(ky13) X*

DA609 *npr-1(ad609) X*

KP6064 *npr-1(ok1447) X*

PR678 *tax-4(p678) III*

CX4544 *ocr-2(ak47) IV*

LSC27 *pdf-1(tm1996) III*

KP6340 *pdfr-1(ok3425) III*

MT6308 *eat-4(ky5) III*

KP0004 *glr-1(n2461) III*

VM487 *nmr-1(ak4) II*

KP6057 *ocr-2(ak47) IV;npr-1(ok1447) X*

KP6058 *ocr-2(ak47) IV;npr-1(ky13) X*

KP6060 *tax-4(p678) III;npr-1(ky13) X*

KP6061 *tax-4(p678) III;npr-1(ok1447) X*

KP6100 *pdf-1(tm1996) III;npr-1(ky13) X*

KP6410 *pdfr-1(ok3425) III;npr-1(ky13) X*

KP6349 *eat-4(ky5) III; npr-1(ky13) X*

CX4978 *kyIs200[sra-6p::VR1, elt-2p::NLS-gfp] (Gift from Cori Bargmann)*

KP6414 *nmr-1(ak4) II; npr-1(ky13) X*

KP6415 *glr-1(n2461) III;npr-1(ky13) X*

VM1123 *dpy-19(n1347) glr-2(ak10) III*

KP6740 *dpy-19(n1347) glr-2(ak10) III; npr-1(ky13) X*

KP7362 npr-1(ky13) X; nuIs439[nlp-12p::GFP]; nuIs519[nlp-12p::ced-3::GFP, vha-6::mCherry]

KP6693 *nuIs472 [pdf-1p::pdf-1::venus, vha-6p::mCherry]*

KP6743 *npr-1(ky13) X; nuIs472*

KP7194 *dpy-19(n1347) glr-2(ak10) III; nuIs472*

KP7195 *dpy-19(n1347) glr-2(ak10) III; npr-1(ky13) X; nuIs472*

AQ906 *bzIs17[mec-4p::YC2.12]*

KP6681 *npr-1(ky13) X; bzIS17*

Strains containing extrachromosomal arrays

CX9396 *npr-1(ad609) X;kyEx1966[flp-21p::npr-1 SL2 GFP, ofm-1p::dsRed] (Gift from Cori Bargmann)*

KP6051 *npr-1(ad609) X;nuEx1519[unc-25p::npr-1::gfp, myo-2p::NLS-mCherry]*

KP6053 *npr-1(ad609) X;nuEx1520[unc-30p::npr-1::gfp, myo-2p::NLS-mCherry]*

KP7149, KP7150 *eat-4(ky5) III; npr-1(ky13) X; nuEx1613-1614[sra-6p::eat-4, myo-2p::NLS-mCherry]*

KP7176, KP7177 *eat-4(ky5) III; npr-1(ky13) X; nuEx1615-1616[sra-9p::eat-4, vha-6p::mCherry]*

KP7198, KP7199 eat-4(ky5) III; npr-1(ky13) X; nuEx1640-1641[mec-4p::eat-4, vha-6p::mCherry]

KP7442 npr-1(ky13) X; nuEx1684[sra-6p::ced-3::GFP, sra-6p::mCherry, vha-6p::mCherry]

KP7633 nuEx1613[sra-6p::eat-4, myo-2p::NLS-mCherry]

KP7634 nuEx1640[mec-4p::eat-4, vha-6p::mCherry]

AQ3304 *ljEx239[sra-6::YC.360]*

KP7353 npr-1(ky13) X; ljEx239

KP7443 npr-1(ky13) X; ljEx239; nuEX1607[flp-21p::npr-1, myo-2p::NLS-mCherry]

KP7495 npr-1(ky13) X; ljEx239; nuEX1683[sra-6p::npr-1, vha-6p::mCherry]

KP7191 dpy-19(n1347) glr-2(ak10) III; npr-1(ky13) X; nuEx1637[nlp-12p::glr-2(gDNA),myo-2p::NLS-mCherry]

KP7192 dpy-19(n1347) glr-2(ak10) III; npr-1(ky13) X; nuEx1638[gcy-28(d)p::glr-2(gDNA),vha-6p::mCherry]

KP7354, KP7355, KP7356 dpy-19(n1347) glr-2(ak10) III; npr-1(ky13) X; nuEx1642-1644[glr-1p::glr-2(gDNA), vha-6p::mCherry]

KP7635 nuEx1637[nlp-12p::glr-2(gDNA),myo-2p::NLS-mCherry]

KP7636 nuEx1638[gcy-28(d)p::glr-2(gDNA),vha-6p::mCherry]

Constructs

eat-4 rescue constructs (sra-6p::eat-4 (KP#2204), sra-9p::eat-4 (KP#2205), and mec-4p::eat-4 (KP#2207)

eat-4 cDNA was amplified by PCR and ligated into expression vectors (pPD49.26) containing the *sra-6* (~3.8kb 5' regulatory sequence: ASH expression), *sra-9* (~3kb 5' regulatory sequence: ASK expression), or *mec-4* (~1.1kb 5' regulatory sequence: Touch neuron expression) promoters.

glr-2 rescue constructs (nlp-12p::glr-2 (KP#2211), gcy-28(d)p::glr-2 (KP#2209), and glr-1p::glr-1 (KP#2208)

glr-2 genomic DNA was amplified by PCR and ligated into expression vectors (pPD49.26) containing the *nlp-12* (~400 bp 5' regulatory sequence: DVA expression), *gcy-28(d)* (~2,9kb 5' regulatory sequence: AIA expression), or *glr-1* (~5.3kb 5' regulatory sequence: ventral cord interneuron (VCI) expression) promoters.

Cell ablation constructs (sra-6p::ced-3::GFP (KP#2151) and nlp-12p::ced-3::GFP (KP#2302)

ced-3 genomic DNA and GFP were amplified by overlapping PCR and ligated into expression vectors (pPD49.26) (using NheI and SacI restriction sites) containing the *sra-6* (~3.8 kb 5' regulatory sequence: ASH expression) or *nlp-12* (~400 bp 5' regulatory sequence: DVA expression) promoters.

Transgenes and germline transformation

Transgenic strains were generated by microinjection of various plasmids with coinjection markers (*myo-2p::NLS-mCherry* (KP#1480) and *vha-6p::mcherry* (KP#1874)). Injection concentration was 40 - 50 ng/µl for all the expression constructs and 10 ng/µl for coinjection markers. The empty vector *pBluescript* was used to bring the final DNA concentration to 100 ng/µl. The *flp-21* promoter (which is expressed in the RMG, ASH, ADL, ASK, URX, and ASI neurons (Macosko et al., 2009)) was used to express transgenes in the RMG circuit.

Lethargus locomotion and behavior analysis

Lethargus locomotion was analyzed as previously described (Choi et al., 2013). Well-fed late L4 animals were transferred to full lawn OP50 bacterial plates. After 1 hour, locomotion of animals in lethargus (determined by absence of pharyngeal pumping) was recorded on a Zeiss Discovery Stereomicroscope using Axiovision software. Locomotion was recorded at 2 Hz for 60 seconds. Centroid velocity of each animal was analyzed at each frame using object-tracking software in Axiovision. Motile fraction of each animal was calculated by dividing the number of frames with positive velocity value with total number of frames. Speed of each animal was calculated by averaging the velocity value at each frame. Quantitative analysis was done using a custom written MATLAB program (Mathworks). Statistical significance was determined using one-way ANOVA with Tukey test for multiple comparisons and two-tailed Student's t test for pairwise comparison.

Adult locomotion and behavior analysis

Locomotion of adult animals was analyzed with the same setup as lethargus locomotion analysis described above, except that well-fed adult animals were monitored 1 – 1.5hr after the transfer to full lawn OP50 bacterial plates. For the capsaicin treatment, 1 day-old animals were transferred to NGM plates containing 50 μM capsaicin (with food), treated with capsaicin for 5 hours, and recorded for their locomotion. Statistical significance was determined using one-way ANOVA with Tukey test for multiple comparisons and two-tailed Student's t test for pairwise comparison.

Cell Ablations

Neurons were ablated in *npr-1(ky13)* mutant worms by transgenes co-expressing CED-3 and a fluorescent protein (GFP or mCherry) under the *sra-6* (ASH ablation) or *nlp-12* (DVA ablation) promoters. ASH or DVA ablations were confirmed after locomotion analysis by fluorescence microscopy.

Aldicarb assay

Sensitivity to aldicarb was determined by analyzing the time course of paralysis following treatment with 1 mM aldicarb (Sigma-Aldrich) as previously described (Nurrish et al., 1999). Briefly, movement of animals was assessed by prodding animals with a platinum wire every 10 minute following exposure to aldicarb. 20-30 animals were tested for each trial. For the capsaicin treatment, adult animals were transferred to NGM plates containing 50 μM capsaicin (with food), treated with capsaicin for 2-3 hours, and assayed for their paralysis on 1 mM aldicarb plates containing 50 μM capsaicin.

Electrophysiology

Electrophysiology was performed on dissected adult worms as previously described (Richmond et al., 1999). Worms were superfused in an extracellular solution containing 127 mM NaCl, 5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 20 mM glucose, 1 mM $CaCl_2$, and 4 mM $MgCl_2$, bubbled with 5% CO_2, 95% O_2 at 20°C. Whole cell recordings were carried out at –60 mV using an internal solution containing 105 mM $CsCH_3SO_3$, 10 mM CsCl, 15 mM CsF, 4mM $MgCl_2$, 5mM EGTA, 0.25mM $CaCl_2$, 10mM HEPES, and 4 mM Na_2ATP, adjusted to pH 7.2 using CsOH. Under these conditions, we only observed endogenous acetylcholine EPSCs. To record GABAergic postsynaptic currents, the holding potential was 0 mV, at which we only observe mIPSCs. All recording conditions were as described (McEwen et al., 2006). To record evoked EPSCs, a 0.4 ms, 30 μA square pulse was applied to a motor neuron cell body with a stimulating electrode placed near the ventral nerve cord (one muscle distance from the recording pipette). Statistical significance was determined using one-way ANOVA with Tukey test for multiple comparisons and two-tailed Student's t test for pairwise comparison.

Fluorescence microscopy and image analysis

Quantitative imaging of coelomocyte fluorescence was performed as previously described (Choi et al., 2013) using a Zeiss Axioskop equipped with an Olympus PlanAPO 100x (NA=1.4) objective and a CoolSNAP HQ CCD camera (Photometrics). Worms were immobilized with 30 mg/ml BDM (Sigma). The anterior coelomocytes were imaged in L4/A lethargus (determined by absence of pharyngeal pumping), and 1 day-old adult animals. Image stacks were captured and maximum intensity projections were

obtained using Metamorph 7.1 software (Universal Imaging). YFP fluorescence was normalized to the absolute mean fluorescence of 0.5 mm FluoSphere beads (Molecular Probes). Statistical significance was determined using Kolmogorov-Smirnov test.

Calcium imaging and analysis

Using Dermabond topical skin adhesive, individual worms were glued to 2% agarose pads in extracellular saline (145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 20 mM D-glucose, and 10 mM HEPES buffer [pH7.2]). To image copper and glycerol responses, single animals were placed in a perfusion chamber (RC-26GLP,Warner Instruments) under a constant flow rate (0.4 ml min^{-1}) of buffer using a perfusion pencil (AutoMate). Outflow was regulated using a peristaltic pump (Econo Pump, Bio-Rad). 10mM $CuCl_2$ (copper(II)chloride dihydrate, Sigma) or 500mM glycerol (Fisher) were delivered using the perfusion, pencil and switch between control and stimulus solutions was done using manually controlled valves. Solutions contained either 10mM $CuCl_2$ in M13 buffer or 500mM glycerol in 40mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 5 mM KPO_4. The stimulus was delivered for 10 seconds starting on the 10^{th} second from the beginning of the movie. Optical recordings were performed on a Zeiss Axioskop 2 upright compound microscope equipped with a Dual View beam splitter and a Uniblitz Shutter. Images were recorded at 10 Hz using an iXon EM camera (Andor Technology) and captured using IQ1.9 software (Andor Technology). For ratiometric imaging, ROI_Y tracked the neuron in the yellow channel, and in the cyan channel, ROI_C moved at a fixed offset from ROI_Y. F was computed as F_Y/F_C following a correction for bleed through. No correction for bleaching was required. Ratio changes were detected and parametrized

using scripts for MATLAB (The Mathworks). Briefly, the scripts average the F value for 5 preceding and including the marked start stimulus frame (F_0) and the 5 frames centered on the marked peak frame (F_1). ΔF was equal to $(F_1 - F_0)/ F_0 \times 100$. Touch-evoked calcium responses in PLM neurons were analyzed as previously described (Choi et al., 2013). Statistical significance was determined using one-way ANOVA with Tukey test for multiple comparisons.

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Chapter 3

FLP-2 and PDF-1 Act in Concert to Arouse *C. elegans* Locomotion

The experiments discussed in this chapter are unpublished work resulting from collaboration between Didi Chen and Kelsey Taylor.

Author Contributions

Didi Chen started this project, originally identifying the role of *frpr-18* and *flp-2*, as well as performing all coelomocyte peptide secretion experiments and testing ASI ablation. Kelsey Taylor performed ASI activation experiments, mouse orexin experiments, *frpr-18* rescue, and the identification of *frpr-18* expressing cells. Qi Hall contributed to the generation of some plasmids. Kelsey Taylor and Joshua Kaplan wrote the chapter, with input from Didi Chen.

Introduction

Animals undergo periods of behavioral quiescence and arousal in response to changes in their environment and metabolic state. Arousal is defined as a state of heightened responsiveness to sensory cues coupled with increased motor activity whereas quiescence is associated with diminished responsiveness and motor activity (Pfaff et al., 2008). Arousal is associated with fear, stress, hunger, and exposure to sexual partners, while quiescence is associated with sleep and satiety (Cirelli, 2009; Pfaff et al., 2008). Quiescence and arousal can persist for minutes to hours. Relatively little is known about the circuit mechanisms that dictate the duration of these behavioral states, nor how transitions between these states are triggered. To address these questions, we have analyzed arousal and quiescence of *C. elegans* locomotion.

During each larval molt, *C.elegans* undergoes a prolonged period of profound behavioral quiescence, termed lethargus behavior, during which locomotion and feeding behaviors are inactive for approximately 2 hours (Cassada and Russell, 1975). Lethargus has properties of a sleep-like state such as reduced sensory responsiveness and homeostatic rebound of quiescence following perturbation (Trojanowski et al., 2015; Trojanowski and Raizen, 2016). Several genes and molecular pathways involved in lethargus behavior have been identified
(Choi et al., 2013; 2015; Monsalve et al., 2011; Nagy et al., 2014a; 2014b; 2013; Nelson et al., 2013; Raizen et al., 2008; Singh et al., 2014; Turek et al., 2013; Van Rompay and Sternberg, 2007). Many sensory responses are diminished during lethargus, including those mediated by a nociceptive neuron (ASH)

(Cho and Sternberg, 2014; Choi et al., 2013; 2015), and by mechanosensory neurons (Choi et al., 2013; Schwarz et al., 2011). Diminished sensory responsiveness during lethargus is likely to be an important circuit mechanism for producing behavioral quiescence (Choi et al., 2013; 2015).

Mutants lacking NPR-1 Neuropeptide Y (NPY) receptors have been utilized as a model for generalized arousal. NPR-1 inhibits the activity of a central sensory circuit (defined by gap junctions to the RMG interneuron) (Macosko et al., 2009). In *npr-1* mutants, sensory responses mediated by the RMG circuit (e.g. avoidance of pheromone, oxygen, and irritant chemicals) are exaggerated, and this heightened acuity is associated with exaggerated locomotion (both during lethargus and in adults) (Cheung et al., 2005; Choi et al., 2013; Gray et al., 2004; Macosko et al., 2009). Mutations that increase (e.g. *npr-1*) and decrease (e.g. *tax-4* CNG and *osm-9* TRPV) RMG circuit activity are associated with locomotion arousal and quiescence respectively (Choi et al., 2013; Coates and de Bono, 2002; de Bono et al., 2002). In *npr-1* mutants, locomotion quiescence during lethargus is nearly completely blocked (Choi et al., 2013; Nagy et al., 2014c). Sensory neurons controlled by the RMG circuit arouse locomotion via secretion of a neuropeptide, pigment dispersing factor (PDF-1), and glutamate (Choi et al., 2013; 2015). These results raise several interesting questions. How are prolonged quiescent and aroused states established by the RMG circuit? Do the different arousing neurotransmitters (i.e. glutamate and PDF-1) interact to stabilize the aroused state? Are there additional transmitters that stabilize the aroused state?

Here we show that locomotion arousal during lethargus is promoted by the concerted action of two arousing neuropeptides (PDF-1 and FLP-2). When animals are

inactive, PDF-1 and FLP-2 secretion is diminished whereas enhanced secretion is associated with aroused locomotion from lethargus. PDF-1 arouses locomotion in part via increased FLP-2 secretion, and vice versa. Thus, locomotion arousal is stabilized by reciprocal positive feedback between PDF-1 and FLP-2. A FLP-2 receptor (FRPR-18) is similar to mammalian orexin/hypocretin receptors and is required for FLP-2's arousing effects. Our results suggest that concerted signaling by two neuropeptides provides a circuit mechanism for synchronized rhythms of behavioral activity.

Results

A neuropeptide receptor (FRPR-18) is required for aroused locomotion during molts in *npr-1* mutants

Locomotion quiescence during the fourth stage larva-to-adult (L4/A) molt is dramatically reduced in *npr-1* mutants (Choi et al., 2013; Nagy et al., 2014c). Mutations inactivating PDF-1 or its receptor (PDFR-1) re-instate a wild-type pattern of molting associated quiescence in *npr-1* mutants. Double mutants lacking both PDF-1 and NPR-1 exhibit a normal pattern of quiescence and arousal during and following molts (Choi et al., 2013), suggesting that changes in PDF-1 signaling are not absolutely required for the rhythmic pattern of quiescence and arousal that is coupled to the molting cycle.

To determine if additional neuropeptides are required for locomotion arousal, we used RNAi to inactivate all predicted neuropeptide receptors in *npr-1* mutants and assayed L4/A locomotion velocity and motile fraction (detailed in methods). One of the genes identified in this screen was *frpr-18*. Inactivating *frpr-18* by either RNAi (Fig. 3.1) or a null mutation (Fig. 3.2) significantly decreased L4/A locomotion velocity and motile fraction of *npr-1* mutants, indicating that molt-associated quiescence was restored. The *frpr-18* null mutation had little effect on the locomotion of *npr-1* adults (Fig. 3.3); however, *frpr-18* single mutant adults exhibited decreased locomotion velocity (Fig. 3.3). A fosmid clone spanning the *frpr-18* genomic locus reinstated the L4/A locomotion quiescence defects in *frpr-18; npr-1* double mutants (Fig. 3.2). Foraging behavior is also altered in *npr-1* mutants, as indicated by an increased tendency of mutant worms to form

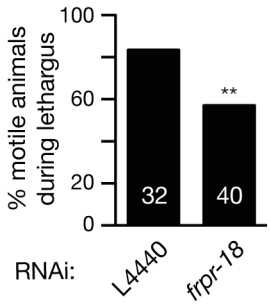

Figure 3.1 RNAi of *frpr-18* suppresses *npr-1* locomotion quiescence defect. The fraction of motile animals during the L4/A lethargus following treatment with RNAi is shown. RNAi was carried out using a RNAi hypersensitive strain (*nre-1 lin-15b*) (Schmitz et al., 2007). Knockdown of *frpr-18* significantly suppressed the npr-1 lethargus locomotion defect, whereas the empty vector control L4440 has no effect. The number of animals analyzed is indicated. Values that differ significantly (by chi-square test) is indicated (**, p<0.01)

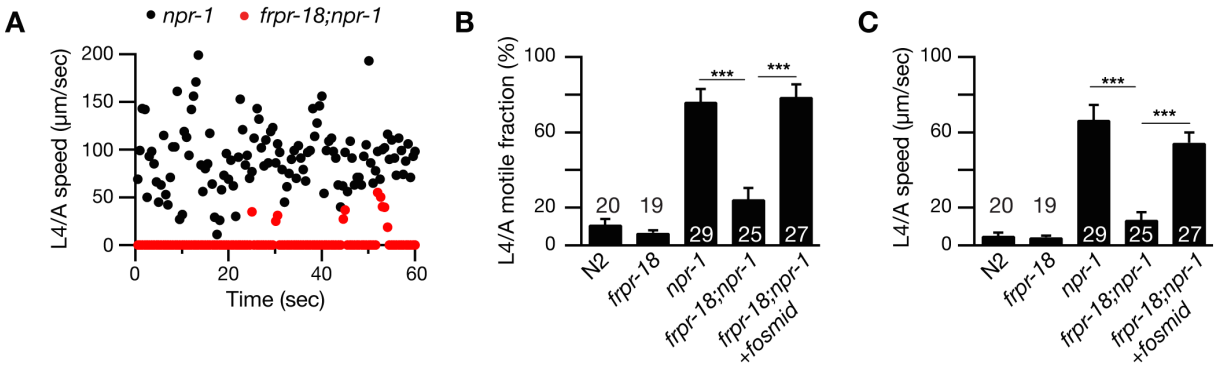

Figure 3.2 FRPR-18 receptors are required for the *npr-1* lethargus defect. Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Instantaneous locomotion velocity (A), average motile fraction (B), and average locomotion velocity (C) are plotted. The *npr-1* L4/A locomotion quiescence defect was suppressed by mutations inactivating FRPR-18 and was reinstated by a fosmid clone containing the frpr-18 genomic locus (B-C) in *frpr-18;npr-1* double mutants. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, p <0.001; ns).

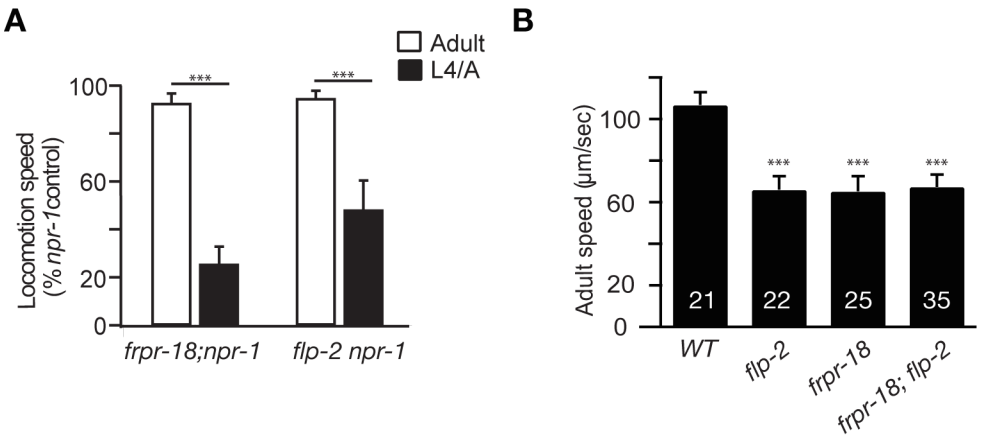

Figure 3.3 *frpr-18* mutation has little effect on *npr-1* adult locomotion. The change in average locomotion velocity (normalized to *npr-1* mutants) is summarized for the indicated genotypes. *frpr-18* mutation decreased *npr-1* mutant locomotion speed during L4/A lethargus by 75% decrease, compared to a 7% decrease in adults. Similarly, locomotion speed during L4/A lethargus was reduced by 52% in *flp-2; npr-1* double mutants, while adult speed was decreased by 5% (A). Locomotion behavior of single adult worms was analyzed in the indicated genotypes and average velocity plotted (B). Both *flp-2* and *frpr-18* single mutants showed reduced locomotion in adult. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, p <0.001).

clumps at the boundaries of the bacterial lawn (de Bono and Bargmann, 1998). This *npr-1* foraging defect was not suppressed in *frpr-18; npr-1* double mutants, indicating the FRPR-18 was not required for other *npr-1* phenotypes (Fig. 3.4). Collectively, these results suggest that FRPR-18 signaling promotes aroused locomotion during lethargus.

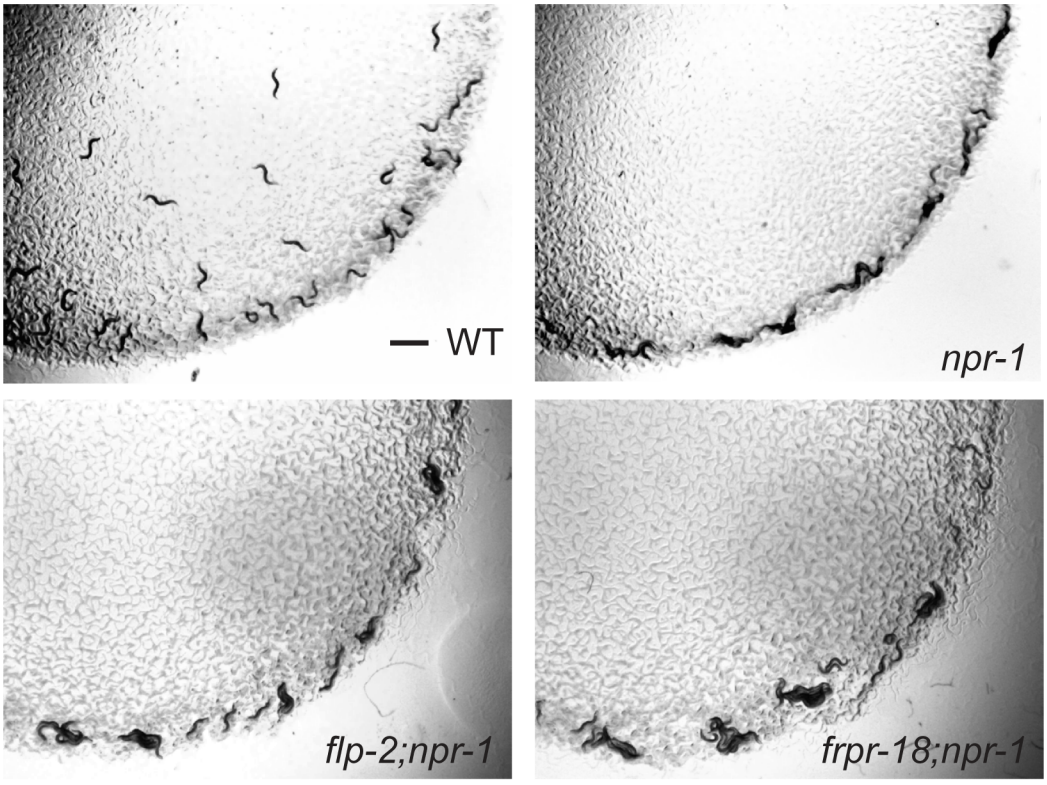

Figure 3.4 Inactivation of *frpr-18* or *flp-2* does not suppress *npr-1* foraging behavior. Representative images of foraging behavior on bacterial lawns are shown for the indicated genotypes. Neither *frpr-18* nor *flp-2* mutations prevented clumping of *npr-1* mutants. Scale bar indicates 1 mm.

An FRPR-18 ligand (FLP-2) also promotes aroused L4/A locomotion in *npr-1* mutants

Prior studies found that two neuropeptides (FLP-2A and B) encoded by the *flp-2* gene activate FRPR-18 receptors expressed in transfected cells (Larsen et al., 2013; Mertens et al., 2005). Prompted by these results, we analyzed the effect of a *flp-2* deletion (*gk1039*) on lethargus locomotion. L4/A locomotion velocity and motile fraction were significantly reduced in *npr-1; flp-2* double mutants compared to *npr-1* single mutants (Fig. 3.5). The *npr-1* quiescence defect could be rescued by restoration of *flp-2*

under its own promoter (Fig. 3.6). The *flp-2* mutation had little effect on *npr-1* adult locomotion, although *flp-2* single mutants exhibited decreased adult locomotion velocity (Fig. 3.3). The *flp-2* mutation also had no effect on the *npr-1* foraging defect (Fig. 3.4), indicating that FLP-2 was not required for other *npr-1* phenotypes.

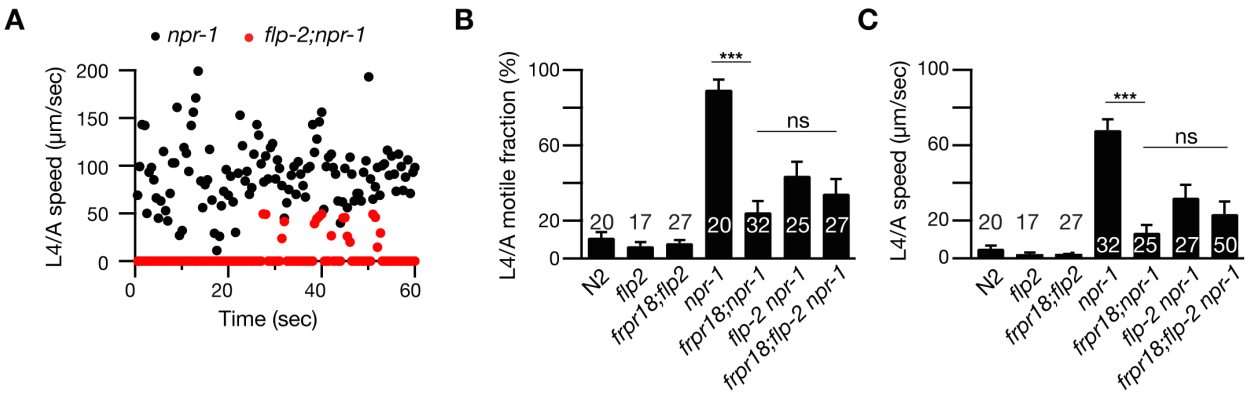

Figure 3.5 FLP-2 peptide is required for the *npr-1* lethargus defect. Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Instantaneous locomotion velocity (A), average motile fraction (B), and average locomotion velocity (C) are plotted. The npr-1 L4/A locomotion quiescence defect was suppressed by mutation inactivating the FRPR-18 ligand, FLP-2 (B-C). The L4/A locomotion velocity and motile fraction of *frpr-18;npr-1* or *flp-2 npr-1* double mutants was not significantly different from *frpr-18;flp-2 npr-1* triple mutants (B-C). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$; ns, not significant).

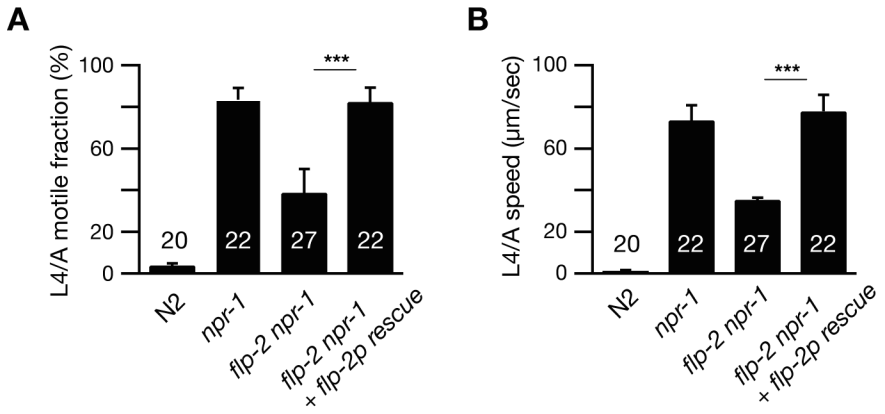

Figure 3.6 *flp-2* can be rescued under its own promoter. Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Average motile fraction (A), and average locomotion velocity (B) are plotted. The *npr-1* L4/A locomotion quiescence defect was restored in *flp-2 npr-1* double mutants by transgene expression of *flp-2* under its own promoter *(flp-2p* rescue*).* The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

If FLP-2 neuropeptides function as FRPR-18 ligands *in vivo* (as predicted by the cell culture data), *flp-2* and *frpr-18* mutations should not have additive effects in double mutants. Consistent with this idea, the L4/A locomotion velocity and motile fraction of *frpr-18; npr-1* double mutants was not significantly different from that observed in *frpr-18; flp-2; npr-1* triple mutants (Fig. 3.5). Similarly, adult locomotion exhibited by *frpr-18; flp-2* double mutants did not significantly differ from that observed in either single mutant (Fig. 3.3). These results indicate that *flp-2* and *frpr-18* function together to arouse locomotion, consistent with FLP-2A/B function as FRPR-18 ligands (Larsen et al., 2013; Mertens et al., 2005).

NPR-1 inhibits FLP-2 secretion during L4/A locomotion quiescence

We previously showed that NPR-1 inhibits PDF-1 secretion during lethargus, and that this effect is required to maintain the molt-associated locomotion quiescence (Choi et al., 2013). Prompted by these results, we tested the idea that NPR-1 also inhibits FLP-2 secretion during lethargus. We analyzed secretion of YFP-tagged FLP-2, expressed by the *flp-2* promoter. Secretion of FLP-2 was assessed by measuring YFP fluorescence in the endolysosomal compartment of coelomocytes, which are specialized scavenger cells that internalize proteins secreted into the body cavity (Fares and Greenwald, 2001). In wild-type animals, FLP-2::YFP secretion was dramatically decreased during the L4/A molt compared to L4 stage animals (Fig. 3.7). This molt-associated decrease in FLP-2::YFP secretion was eliminated in *npr-1* mutants, suggesting that NPR-1 inhibits FLP-2 secretion during molts and that decreased FLP-2 secretion is required for molt-associated locomotion quiescence.

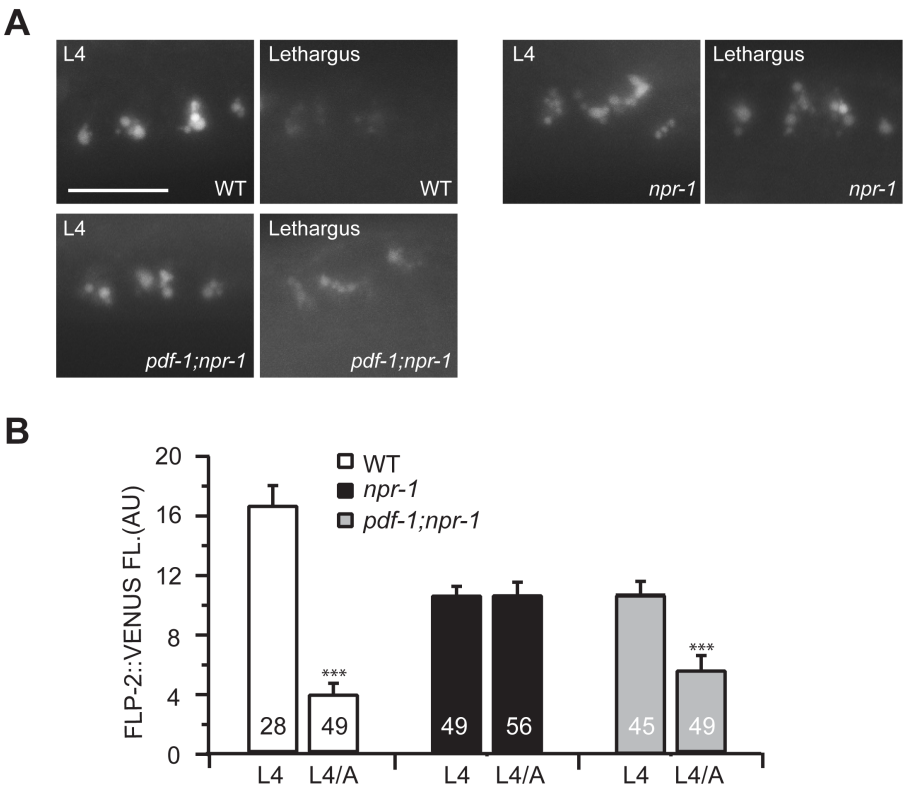

Figure 3.7 NPR-1 inhibits FLP-2 secretion during L4/A locomotion quiescence. FLP-2 secretion was analyzed in the indicated genotypes. VENUS-tagged FLP-2 was expressed with the *flp-2* promoter. Representative images (A) and summary data (B) are shown for coelomocyte fluorescence in L4 and L4/A animals of the indicated genotypes. FLP-2:: VENUS coelomocyte fluorescence was dramatically reduced during the L4/A lethargus of wild type animals, but not in *npr-1* mutants. *pdf-1;npr-1* double mutants exhibited decreased FLP-2 secretion during lethargus. Scale bar indicates 10 μm. Values that differ significantly from L4 controls (B) are indicated (***, p <0.001).

ASI neurons promote arousal

FLP-2 is expressed in several neurons in head ganglia, including AIA, ASI, MC, M4, I5, and RID (Kim and Li, 2004) (Fig. 3.8). To further characterize the cellular mechanism for FLP-2's arousing effects, we identified cells that express *frpr-18*. An *frpr-*

18 promoter construct expressed GFP in many neurons (including AIY, ASI, BAG, URA, CAN, I6, PVQ, RIM, DVA and VC) and in the anal sphincter muscle (Fig. 3.9).

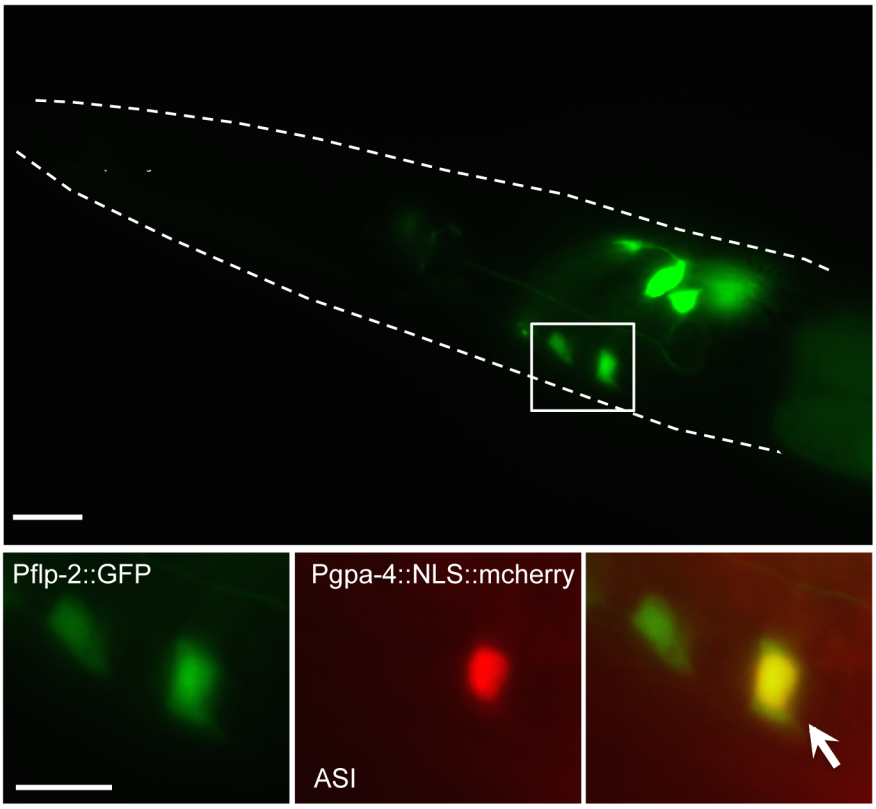

Figure 3.8 *flp-2* is expressed in several head neurons including ASI. Expression of *flp-2* is observed in head sensory neurons, including ASI. P*flp-2* ::GFP was detectable in several head neurons. The localization of *flp-2* in the chemosensory ASI neuron is identified by the co-localization between P*flp-2* ::GFP and the ASI marker P*gpa-4*:: mcherry (A). Scale bar indicates 10 μm.

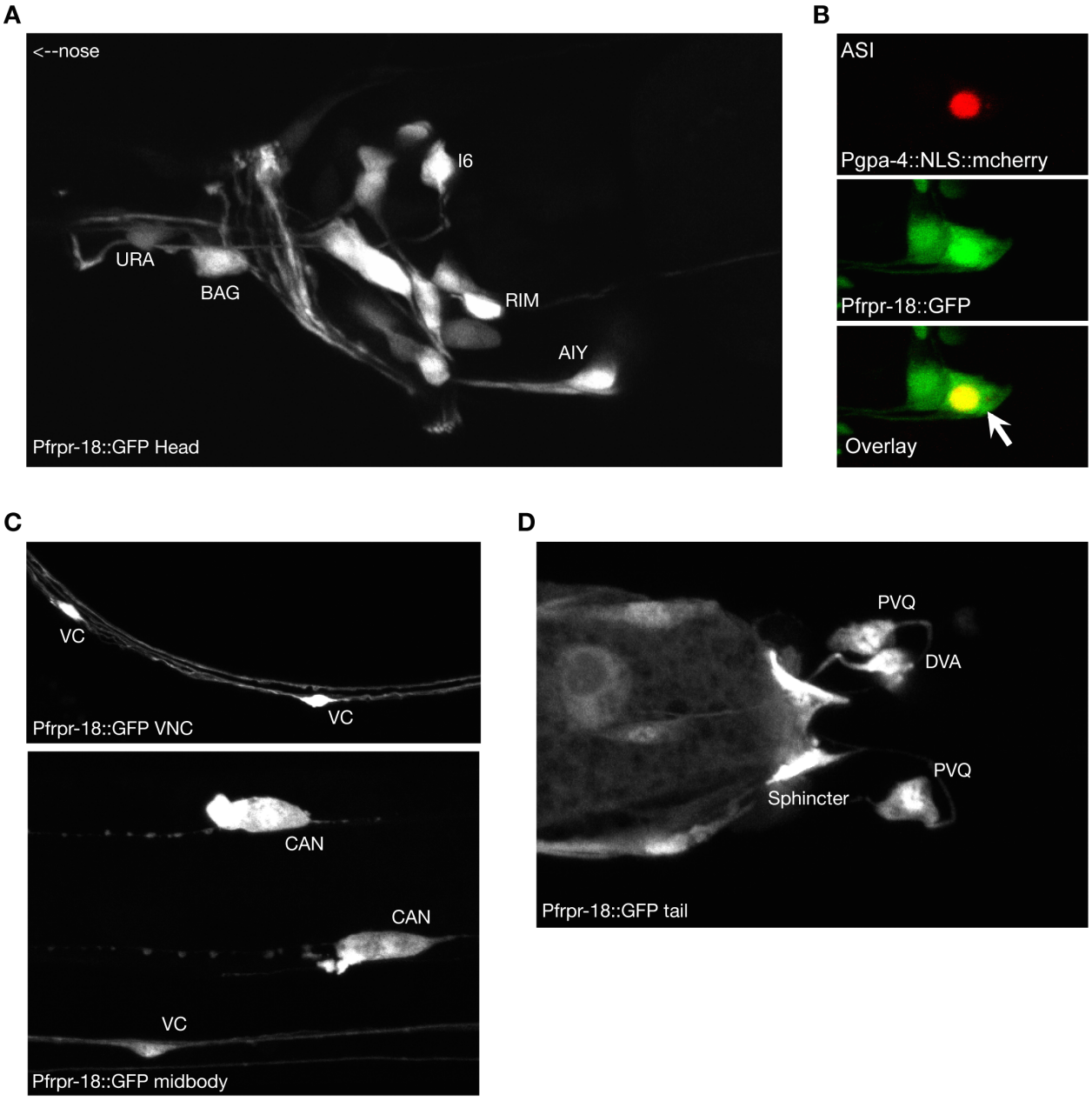

Figure 3.9 *frpr-18* is expressed in a subset of neurons in the head, midbody, and tail. Transgenic hermaphrodite animals expressing a GFP (green fluorescent protein) reporter construct under control of the *frpr-18* promoter were analyzed. *frpr-18*p:GFP is expressed in URA, BAG, I6, RIM, AIY neurons in the head of the worm (A). *frpr-18*p expression was confirmed in the ASI neurons by co-expression with red fluorescent protein under the ASI specific promoter, *gpa-4*. (B). In the ventral nerve cord and midbody of adult animals, *frpr-18*p:GFP is expressed in the VC and CAN neurons (C). In the tail, *frpr-18*p:GFP was identified in the anal sphincter muscle, and PVQ and DVA neurons (D).

ASI neurons co-express FLP-2 (Kim and Li, 2004) (Fig. 3.7), FRPR-18 (Fig. 3.8), and PDF-1 (Barrios et al., 2012; Janssen et al., 2009), suggesting that these neurons play an important role in arousal. To test this idea, we genetically ablated ASI neurons in *npr-1* mutants. ASI cell death was induced by a transgene that expresses a pro-apoptotic caspase (CED-3) and GFP in ASI neurons (using the *str-3* promoter). ASI cell death was confirmed by the absence of GFP fluorescence in transgenic animals. The L4/A motile fraction and locomotion rate of *npr-1* worms were significantly reduced in animals lacking ASI neurons (Fig. 3.10).

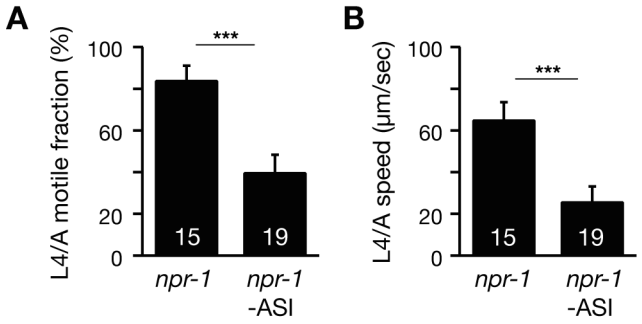

Figure 3.10 ASI neurons promote arousal. Locomotion behavior of single worms during the L4/A lethargus was analyzed in the *npr-1* worms whose ASI neurons were genetically ablated by transgenic overexpression of CED-3 in ASI (*str-3* promoter) (A-B). The *npr-1* L4/A locomotion quiescence defect was suppressed by ASI ablation. Average motile fraction (A), and average locomotion velocity (B) are plotted. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

To determine if artificially activating ASI neurons arouses locomotion, we analyzed transgenic animals expressing rat TRPV1 capsaicin receptors in ASI. A 5-hour capsaicin treatment had no effect on L4/A motile fraction or locomotion velocity (Fig.

3.11). These results suggest that ASI activation with capsaicin is not sufficient to arouse locomotion during molts, and that the arousing effects of FLP-2/FRPR-18 may require activation of multiple neurons other than ASI.

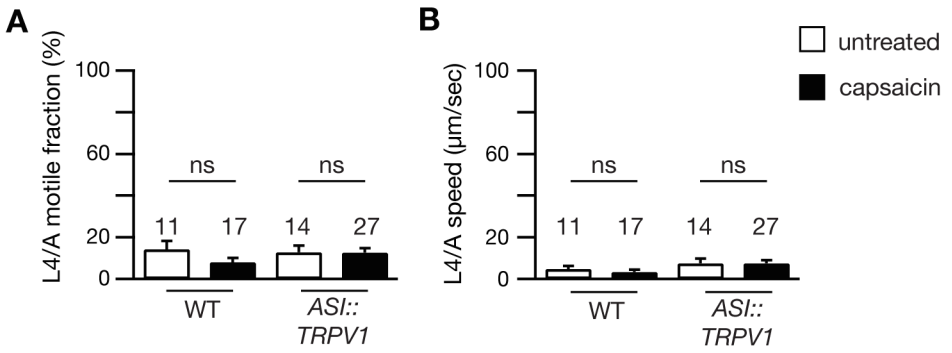

Figure 3.11 Forced depolarization of ASI has no effect on L4/A locomotion quiescence. Rat TRPV1 was ectopically expressed in ASI neurons (using the *gpa-4* promoter). Locomotion behavior of wild type or transgenic worms during the L4/A lethargus was analyzed with or without 50 µM capsaicin treatment (4-5 hr). Average motile fraction (A) and locomotion velocity (B) are plotted. Capsaicin treatment had no effect on either motile fraction or velocity of wild type or transgenic animals. The number of animals analyzed is indicated for each genotype. Significance is indicated (ns, not significant).

FLP-2 and PDF-1 jointly promote arousal by reciprocal positive feedback

Thus far, our results suggest that NPR-1 inhibits secretion of two arousal neuropeptides, FLP-2 and PDF-1 (Choi et al., 2013), thereby promoting quiescence. FLP-2 and PDF-1 could act independently to arouse locomotion or they could comprise components of a single arousal pathway. If they function independently, *flp-2* and *pdf-1* mutations should have additive effects on locomotion in double mutants. Contrary to this idea, inactivating FLP-2 or its receptor (FRPR-18) did not further enhance the reduced

L4/A locomotion of *pdf-1; npr-1* double mutants, nor did these mutations enhance the reduced adult locomotion defect of *pdfr-1* single mutants (Fig. 3.12). These results support the idea that *frpr-18*, *flp-2*, and *pdf-1* likely act in a single genetic pathway to mediate arousal.

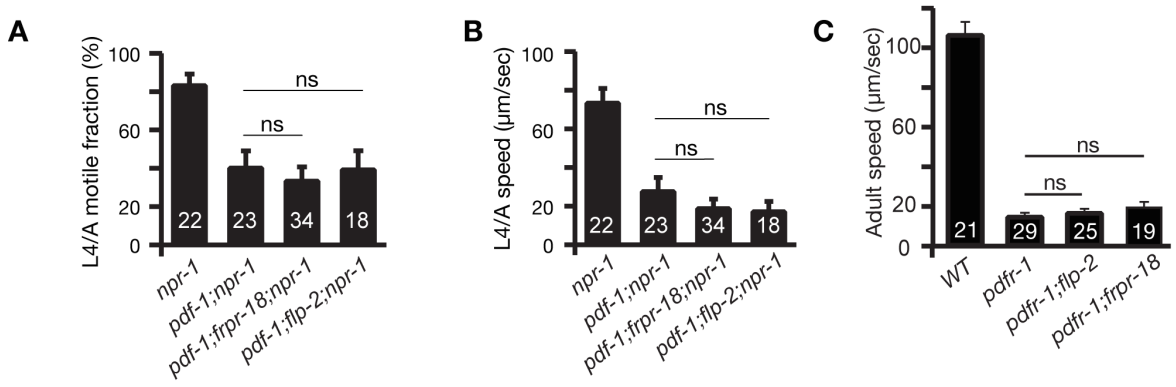

Figure 3.12 FLP-2, FRPR-18, and PDF-1 act in a single genetic pathway. Locomotion behavior of single worms during the L4/A lethargus (A-B) and adults (C) was analyzed in the indicated genotypes. Inactivation of FRPR-18 or FLP-2 did not further decrease the locomotion activity of *pdf-1;npr-1* mutants during the L4/A lethargus. Average motile fraction (A), and average locomotion velocity (B) are plotted. In addition, *flp-2* or *frpr-18* mutation didn't further decrease the locomotion activity of *pdfr-1* adult worms (C). Values that differ significantly are indicated (ns, not significant). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM.

Given their function in a single genetic pathway, we next asked if FLP-2 promotes PDF-1 secretion. To test this idea, we analyzed PDF-1::YFP secretion in *flp-2* mutants. In wild-type animals, PDF-1::YFP secretion is inhibited during the L4/A molt and this effect was eliminated in *npr-1* mutants, as shown in our prior study (Choi et al., 2013). Inhibition of PDF-1::YFP secretion during lethargus was restored in both *flp-2; npr-1* and in *frpr-18; npr-1* double mutants (Fig. 3.13). These results suggest that FRPR-

18 and FLP-2 stimulate PDF-1 secretion, which could contribute to FLP-2's arousing effects. Analogous experiments suggest that PDF-1 promotes FLP-2 secretion (Fig. 3.7). These results suggest that FLP-2 and PDF-1 secretion are regulated by reciprocal positive feedback. Positive feedback between FLP-2 and PDF-1 could provide a mechanism for stabilizing (or prolonging) aroused locomotion in *npr-1* mutants.

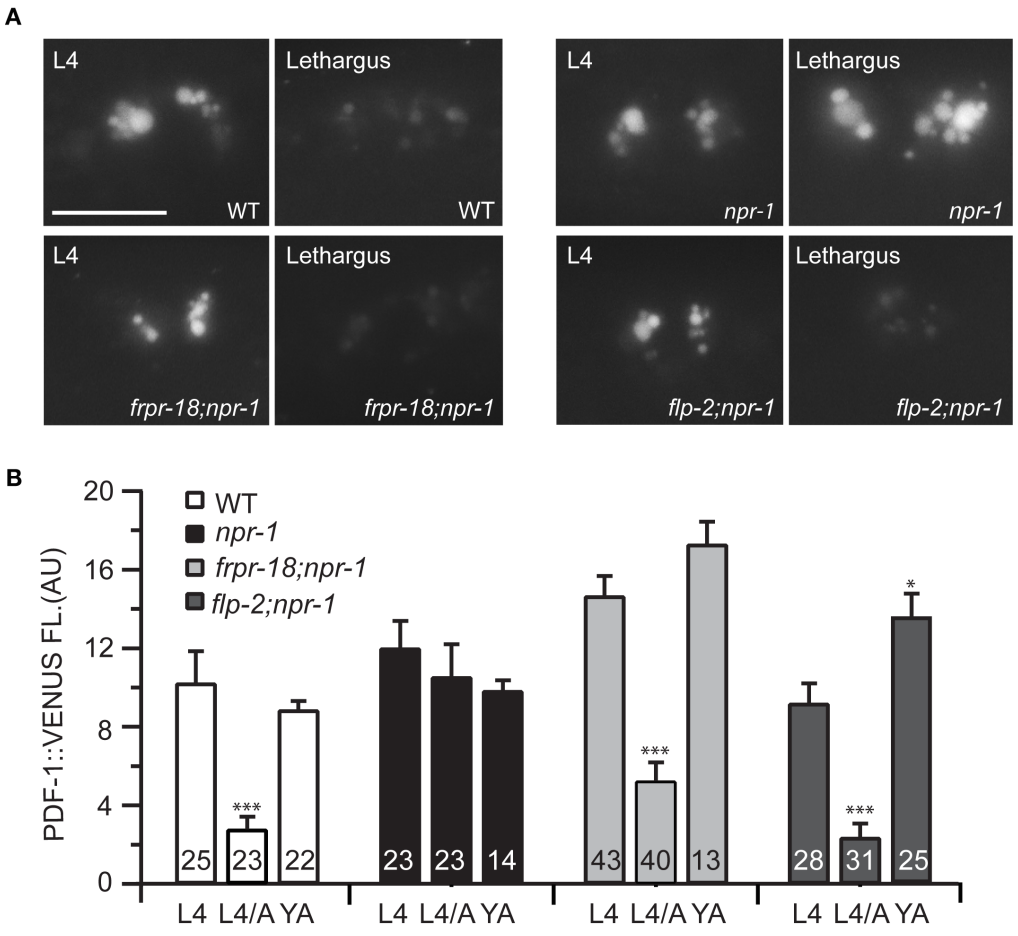

Figure 3.13 FLP-2 and PDF-1 jointly promote arousal by reciprocal positive feedback. PDF-1 secretion was analyzed in the indicated genotypes. VENUS-tagged PDF-1 was expressed with the *pdf-1* promoter. Representative images (A) and summary data (B) are shown for coelomocyte fluorescence in L4, L4/A, and young adults (8 hours post L4) of the indicated genotypes. PDF-1:: VENUS

Figure 3.13 (Continued). coelomocyte fluorescence was dramatically reduced during the L4/A lethargus of wild type animals, but not in *npr-1* mutants, as previously reported (Choi et al., 2013). *frpr-18;npr-1* and *flp-2;npr-1* double mutants exhibited decreased PDF-1 secretion during lethargus. Scale bar indicates 10 μm. Values that differ significantly from L4 controls (D) are indicated (***, $p < 0.001$; ns, not significant). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM.

FLP-2 and FRPR-18 are functionally analogous to vertebrate orexin and orexin receptors

The neuropeptide orexin promotes wakefulness and feeding in vertebrates. Orexin orthologs have not been described in invertebrates, suggesting that their arousal is mediated by a distinct mechanism. Interestingly, FRPR-18 has significant similarity to mammalian orexin type 2 receptors (mOxR2) (BLAST score: E=1e-08), implying that FRPR-18 and mOxR2 receptors could perform analogous functions. To test this idea, we asked if a transgene expressing the mouse mOxR2 receptor could rescue the *frpr-18* mutant defect. Consistent with this idea, an mOxR2 transgene (expressed by the *frpr-18* promoter) re-instated the L4/A locomotion quiescence defect in *frpr-18;npr-1* double mutants (Fig. 3.13A-B). Similarly, expressing mOxR2 receptors only in ASI neurons (using the *gpa-4* promoter), restored the *npr-1* L4/A locomotion quiescence defect to *frpr-18;npr-1* animals (Fig. 3.14C-D). Thus, mOxR2 expression compensates for the absence of FRPR-18 receptors, supporting the idea that FRPR-18 and orexin receptors perform analogous functions in arousal.

To test the idea that FLP-2 is functionally analogous to vertebrate orexins, we analyzed the effect of *flp-2* mutations on the rescuing activity of the mOxR2 transgene.

We found that inactivating FLP-2 blocked the ability of mOxR2 to promote aroused L4/A locomotion in *frpr-18; npr-1* double mutants (Fig. 3.14A-B). This result suggests that endogenously expressed FLP-2 neuropeptides are required for mOxR2's arousing effects. Taken together, these results suggest that FLP-2 is functionally analogous to mammalian orexin and can activate either the FRPR-18 or mOxR2 receptor.

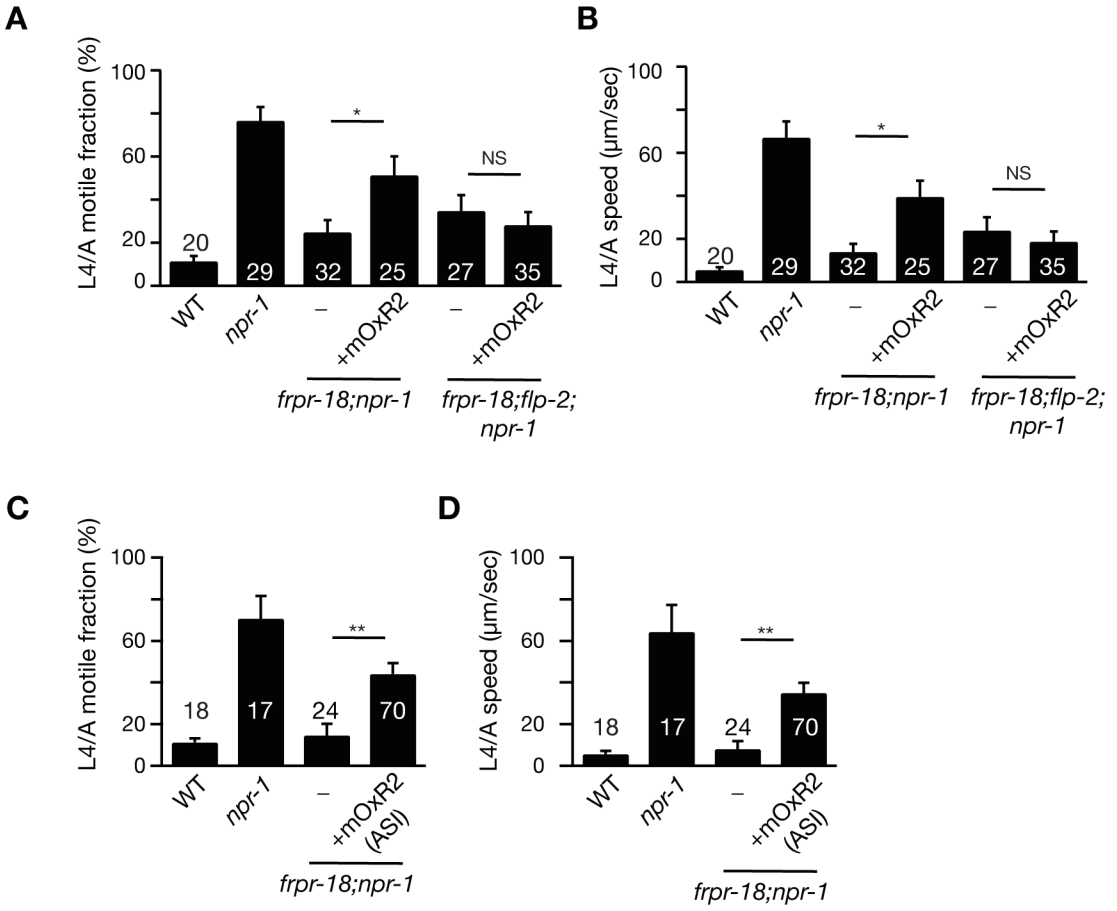

Figure 3.14 FLP-2 and FRPR-18 are functionally analogous to vertebrate orexin and orexin receptors. Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. The *npr-1* locomotion quiescence defect was reinstated in *frpr-18;npr-1* double mutants by transgenes expressing mouse OX2R with the *frpr-18* promoter (A-B) or the ASI-specific promoter *gpa-4* (C-D). The transgene expressing mouse OX2R under the

Figure 3.14 (Continued). *frpr-18* promoter did not reinstate the *npr-1* locomotion quiescence defect in *frpr-18;flp-2 npr-1* triple mutants (A-B). Average motile fraction (A,C), and average locomotion velocity (B,D) are plotted. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$; **, $p < 0.01$).

Discussion

Neuropeptides play a pivotal role in promoting arousal and wakefulness across phylogeny. It was previously thought that different arousing peptides were utilized in vertebrates and invertebrates. While orexin is known to promote wakefulness in vertebrates (including humans, dogs, mice, and zebrafish), PDF promotes wakefulness in invertebrates (including flies and worms) (Chemelli et al., 1999; Choi et al., 2013; Lin et al., 1999; Nishino et al., 2000; Renn et al., 1999). Here we identify FLP-2 as a new arousing neuropeptide in *C. elegans*, and we show that its arousing effects are mediated by an orexin-like receptor, FRPR-18. Our results suggest that the invertebrate *C. elegans* may utilize an orexin-like system to stabilize the aroused state with similar mechanisms as found in vertebrates.

Several results support the idea that FLP-2 promotes locomotion arousal during lethargus. Mutations inactivating FLP-2 or FRPR-18 restore molt-associated quiescence to *npr-1* mutants. FLP-2 secretion is inhibited during molts (when locomotion is quiescent) and this inhibition is blocked in *npr-1* mutants (which lack molt-associated locomotion quiescence). Thus, locomotion quiescence and arousal are linked to decreased and increased FLP-2 signaling respectively.

FRPR-18 has significant sequence homology to vertebrate OXR2 receptors, suggesting that the *flp-2* encoded peptides (FLP-2A and B) are functionally analogous to vertebrate orexins. To further test this idea, we showed that mOXR2 expression rescues that *frpr-18* mutant defect in locomotion arousal and that this rescuing activity requires expression of endogenous FLP-2 peptides. These results support the idea that FRPR-18 and mOXR2 are functional analogs and that FLP-2A/B peptides may activate mOXR2 receptors. These results are surprising because FLP-2A/B are RFamide peptides (defined by their carboxy-terminal RF motifs) while Orexin A and B have conserved c-terminal sequences lacking the RF motif.

We also show that ASI neurons play an important role in promoting locomotion arousal. Three arousal-inducing genes (*pdf-1*, *flp-2*, and *frpr-18*) are expressed in ASI. A transgene expressing mOxR2 in ASI re-instated the lethargus quiescence defect in *frpr-18; npr-1* double mutants, suggesting that FRPR-18 acts in ASI to promote arousal. Genetic ablation of ASI significantly decreased locomotion arousal during molts in *npr-1* mutants. However, forced activation of ASI neurons with capsaicin was not sufficient to arouse locomotion during molts. FRPR-18 is expressed in several other neurons that were previously implicated in controlling locomotion including RIM and AIY (Alkema et al., 2005; Flavell et al., 2013; Gray et al., 2005; Piggott et al., 2011). These results suggest that FLP-2's arousing effects are mediated by multiple neurons.

Mutations disrupting orexin or orexin receptors are associated with narcolepsy in humans, dogs, and mice. In orexin and mOxR2 knockout mice, the total amount of sleep is unaltered; however, the duration of awake bouts is dramatically reduced (Mochizuki et al., 2004; Willie et al., 2003). This fragmentation of wakefulness in narcoleptic mice has

been interpreted to mean that orexin stabilizes wakefulness. Our results support two mechanisms for stabilizing aroused states. First, FLP-2 and FRPR-18 are co-expressed in ASI neurons, thereby producing an autocrine positive feedback loop for ASI activation. Second, we find that FLP-2 and PDF-1 secretion are regulated by reciprocal positive feedback, i.e. FLP-2 promotes PDF-1 secretion and vice versa. We propose that these circuit motifs stabilize the aroused state. Interestingly, both motifs are conserved in mammalian arousal circuits. Mouse orexin directly activates orexin expressing neurons via activation of mOxR2 receptors (Yamanaka et al., 2010). Orexin also activates neurons that express several other arousing neurotransmitters (e.g cholinergic, histaminergic, noradrenergic, and serotonergic neurons) (Brown et al., 2012). Thus, autocrine positive feedback and reciprocal positive feedback are conserved motifs found in arousal circuits.

Last, our results show that NPR-1 inhibits FLP-2 secretion. A similar link between NPY and orexin has also been found in rodents. NPY inhibits orexin positive neurons in the hypothalamus (Fu, 2004) and orexin inhibits NPY positive neurons in the thalamus (Palus et al., 2015). Reciprocal inhibition between arousing and quiescence neurons is proposed to be a mechanism for creating bistability of sleep and wake states. If reciprocal inhibition is conserved in *C. elegans*, we expect that the arousing peptides (FLP-2 and PDF-1) would inhibit cells expressing quiescence promoting ligands (FLP-11, FLP-18, FLP-21, and NLP-22) (Choi et al., 2013; Nelson et al., 2013; Turek et al., 2016).

To conclude, in this study we identified a new arousing neuropeptide, FLP-2, that regulates *C. elegans* molting-associated quiescence through an orexin-like receptor,

FRPR-18. FLP-2 acts in concert with PDF-1 through reciprocal positive feedback to promote arousal. The many mechanistic parallels between mammalian orexin and FLP-2/FRPR-18 suggest that *C. elegans* may be a powerful genetic invertebrate system to study the role of orexin in behavioral arousal.

Materials and Methods

Strains

Strain maintenance and genetic manipulation were performed as described (Brenner, 1974). Animals were cultivated at 20°C on agar nematode growth media (NGM) seeded with OP50 (for imaging and behavior) or HB101 *E.coli* (for electrophysiology). Wild type reference strain was N2 Bristol. Strains used in this study are as follows:

Mutant strains and integrants

KP6048 *npr-1(ky13) X*

KP7147 *frpr-18(ok2698) V*

KP7380 *flp-2*(gk1039) *X*

KP7487 *frpr-18(ok2698) V;flp-2(gk1039) X*

KP7918 *frpr-18(ok2698) V;npr-1(ky13) X*

KP7420 *flp-2(gk1039) npr-1(ky13) X*

KP7488 *frpr-18(ok2698) V;flp-2(gk1039) npr-1(ky13) X*

KP7422 *nuIs513[flp-2p::flp-2::venus, vha-6p::mCherry]*

KP7435 *npr-1(ky13) X;nuIs513*

KP7398 *pdf-1(tm1996);npr-1(ky13);nuIs513*

LSC27 *pdf-1(tm1996) III*

KP6340 *pdfr-1(ok3425) III*

KP6100 *pdf-1(tm1996) III;npr-1(ky13) X*

KP6410 *pdfr-1(ok3425) III;npr-1(ky13) X*

KP7385 *pdfr-1(ok3425) III;frpr-18(ok2698) V*

KP7323 *pdfr-1(ok3425) III; flp-2(gk1039) X*

KP7384 *pdfr-1(ok3425) III;frpr-18(ok2698) V;npr-1(ky13) X*

KP7399 *pdfr-1(ok3425) III; flp-2(gk1039) npr-1(ky13) X*

KP7393 *pdf-1(tm1996) III; flp-2(gk1039) npr-1(ky13) X*

KP7384 *pdf-1(tm1996) III;frpr-18(ok2698) V;npr-1(ky13) X*

KP6693 *nuIs472 [pdf-1p::pdf-1::venus, vha-6p::mCherry]*

KP6743 *npr-1(ky13) X; nuIs472*

KP7388 *frpr-18(ok2698) III;nuIs472*

KP7389 *flp-2(gk1039) X;nuIs472*

Strains containing extrachromosomal arrays

KP7587-7589 *frpr-18(ok2698) III; npr-1(ky13) X; nuEx1689-1691[WRM0630bG11 fos;vha-6p::mCherry]*

KP7417 *nuEx1648[flp-2p::gfp,vha-6p::mCherry]*

KP7414 *nuEx1649[frpr-18p::GFP, flp-6p::NLS-mCherry, vha-6p::mCherry]*

KP7500 *nuEx1685[frpr-18p::GFP, gpa-4p::NLS-mCherry]*

KP7502 *nuEx1687[frpr-18p::GFP, flp-6p::NLS-mCherry]*

KP7562 *nuEx1688[frpr-18p::GFP, sra-6p::mCherry]*

KP7982 *nuEx1747[frpr-18p::GFP];otIs518[eat-4(fosmid::SL2::mCherry::H2B + (pBX)pha-1(+)] III; him-5(e1490) V*

KP7983 *nuEx1747[frpr-18p::GFP];otIs544[cho-1(fosmid::SL2::mCherry::H2B + (pBX)pha-1(+)] III; him-5(e1490) V*

KP7431-KP7434 *npr-1(ky13);nuEx1656-1659[str-3p::ced-3::GFP, daf-7p::GFP, vha-6p::mCherry]*

KP7426 *flp-2(gk1039) npr-1(ky13) X; nuEx1652[flp-2p::flp-2(gDNA), vha-6p::mCherry]*

KP77425 *frpr-18(ok2698) III; npr-1(ky13) X; nuEx1651[frpr-18p::mOx2R, vha-6p::mCherry]*

KP7593 *frpr-18(ok2698) III; flp-2(gk1039) npr-1(ky13) X; nuEx1651*

KP7890-7891 *frpr-18(ok2698) III; npr-1(ky13) X; nuEx1736-1737[gpa-4p::mOx2R, vha-6p::mCherry]*

Constructs

FLP-2 secretion construct (flp-2p::flp-2::VENUS (KP#2282))

flp-2 genomic DNA and YFP (VENUS) containing a stop codon were each amplified by PCR and ligated into the expression vector pPD49.26 (Addgene) containing the *sra-6* (~3.8kb 5' regulatory sequence: ASH expression) promoters.

frpr-18 and flp-2 expression constructs (frpr-18p::GFP (KP#2276) and flp-2p::GFP (KP#2271))

DNA corresponding to the *frpr-18* regulatory sequence (~ 2.6 kb 5') or *flp-2* regulatory sequence (~ 3 kb 5') was amplified by PCR and ligated into expression vectors (pPD95.75) containing GFP coding sequence.

flp-2 rescue constructs (KP#2283)

flp-2 genomic DNA (with stop codon) was amplified by PCR and ligated into the expression vectors (PD49.26) containing the *flp-2* promoter (~ 3 kb 5' regulatory sequence).

ASI cell ablation construct (str-3p::ced-3::GFP (KP#2150))

ced-3 cDNA and GFP were amplified by overlapping PCR and ligated into expression vectors (pPD49.26) (using NheI and SacI restriction sites) containing the *str-3* (~3 kb 5' regulatory sequence: ASI expression) promoter.

Mouse orexin receptor 2 constructs (frpr-18p::mOxR2 (KP#2290) and gpa-4p::mOxR2 (KP#3251))

cDNA of mouse orexin type 2 receptor (mOxR2) (~1.3 kb) was amplified by PCR from a mouse cDNA library and ligated into expression vectors (pPD49.26) (using NheI and NcoI restriction sites) containing the *frpr-18* promoter (~2.6 kb 5' regulatory sequence) or *gpa-4* promoter (~3 kb 5' regulatory sequence: ASI expression)

Transgenes and germline transformation

Transgenic strains were generated by microinjection of various plasmids with coinjection markers (*myo-2p::NLS-mCherry* (KP#1480) and *vha-6p::mcherry* (KP#1874)). Injection concentration was 40 - 50 ng/µl for all the expression constructs and 10 ng/µl for coinjection markers. The empty vector *pBluescript* was used to bring the final DNA concentration to 100 ng/µl.

Lethargus locomotion and behavior analysis

Lethargus locomotion was analyzed as previously described (Choi et al., 2013). Well-fed late L4 animals were transferred to full lawn OP50 bacterial plates. After 1 hour, locomotion of animals in lethargus (determined by absence of pharyngeal pumping) was recorded on a Zeiss Discovery Stereomicroscope using Axiovision software. Locomotion was recorded at 2 Hz for 60 seconds. Centroid velocity of each animal was analyzed at each frame using object-tracking software in Axiovision. Motile fraction of each animal was calculated by dividing the number of frames with positive velocity value with total number of frames. Speed of each animal was calculated by averaging the velocity value at each frame. For ASI activation experiments, early L4 animals were transferred to NGM plates containing 50 μM capsaicin (with food) and treated with capsaicin for 4-5 hours. Quantitative analysis was done using a custom written MATLAB program (Mathworks). Statistical significance was determined using one-way ANOVA with Tukey test for multiple comparisons and two-tailed Student's t test for pairwise comparison.

Adult locomotion and behavior analysis

Locomotion of adult animals was analyzed with the same setup as lethargus locomotion analysis described above, except that well-fed adult animals were monitored 5-10 min after the transfer to full lawn OP50 bacterial plates. Foraging behavior was analyzed as described (de Bono and Bargmann, 1998). Briefly, approximately 150 well-fed adult animals were placed on NGM plates seeded with 200 μl OP50 E.coli 2 days before the assay. After 3 hours, images were taken for each genotype. Statistical significance was

determined using one-way ANOVA with Tukey test for multiple comparisons and two-tailed Student's t test for pairwise comparison.

RNAi screen

A small-scale RNAi feeding screen was performed as described (Kamath et al., 2003). The screen was performed in the neuronal RNAi hypersensitive mutant background (*nre-1 lin-15b; npr-1(ky13*) (Schmitz et al., 2007). 114 neuropeptide receptor genes were selected for the screen (Frooninckx et al., 2012). After 5 days of RNAi treatment (2 generation) at 20°C, well-fed late L4 animals were transferred to full lawn OP50 bacterial plates. After 1 hour, animals in lethargus (determined by absence of pharyngeal pumping) were scored for their motility. Statistical significance was determined using chi-square test.

Cell ablations

Neurons were ablated in *npr-1(ky13)* mutant worms by transgenes co-expressing CED-3 and a fluorescent protein (GFP or mCherry) under the *str-3* (ASI ablation) promoter. ASI ablation was confirmed by fluorescence microscopy.

Fluorescence microscopy and image analysis

Quantitative imaging of coelomocyte fluorescence was performed as previously described (Choi et al., 2013) using a Zeiss Axioskop equipped with an Olympus PlanAPO 100x (NA=1.4) objective and a CoolSNAP HQ CCD camera (Photometrics). Worms were immobilized with 30 mg/ml BDM (Sigma). The anterior coelomocytes were imaged in L4/A lethargus (determined by absence of pharyngeal pumping), and 1 day-old

adult animals. Image stacks were captured and maximum intensity projections were obtained using Metamorph 7.1 software (Universal Imaging). YFP fluorescence was normalized to the absolute mean fluorescence of 0.5 mm FluoSphere beads (Molecular Probes). Statistical significance was determined using Kolmogorov-Smirnov test.

Identification of FRPR-18 expressing neurons

GFP-expressing cells of *frpr-18*p::GFP were evaluated based on their position, morphology, and projection pattern. Identification of many neurons was made by analysis of *frpr-18*p::GFP co-expression with RFP (mCherry) driven constructs under promoters with known expression patterns. These include *gpa-4*p::mCherry for ASI, *flp-6*p::mCherry for I6 (and to eliminate ASE neurons), *sra-6*p::mCherry for PVQ neurons (and to eliminate ASH neurons). Several identifications were made by comparing co-expression of *frpr-18*p::GFP with *eat-4*p::RFP (Serrano-Saiz et al., 2013) and *cho-1*p::RFP (O. Hobert, personal communication) known expression patterns. Images were taken using a 60x objective (NA 1.45) on a Olympus FV-1000 confocal microscope. Maximum intensity projections of Z-series stacks were made using Metamorph 7.1 software (Molecular Devices, Sunnvale, CA, US).

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Chapter 4

Concluding Remarks and Future Directions

The experiments discussed in this chapter are unpublished work. Kelsey Taylor performed all experiments.

Implications of the regulation of behavioral arousal by NPR-1

Organisms undergo changes in behavior in response to various environmental and developmental cues. Our study investigated arousal utilizing the molting-associated behavioral quiescent state in *C. elegans*, lethargus. We've demonstrated that *npr-1* mutants can be used as a model for heightened arousal from lethargus, and in doing so have identified a number of neuropeptides and neurons which modulate quiescence. These studies provide insight on 1) the broad network of sensory neurons that contribute to arousal, 2) the modulation of sensory-motor circuitry for inducing quiescence and 3) the conservation of neurotransmitters in the regulation of behavioral arousal across phylogeny.

A broad network of sensory neurons contribute to arousal

Our findings suggest that a broad network of sensory neurons arouse locomotion during quiescence. We've found that mechanosensory neurons (ALM and PLM), nociceptive neurons (ASH), PDF-1 expressing neurons (ASK), stretch sensitive neuron (DVA), and chemosensory neurons (ASI) all contribute to behavioral arousal (Choi et al., 2013; Schwarz et al., 2011; Chapter 2; Chapter 3). For the following reasons, our findings suggest that sensory activity is decreased during quiescence:

1) Sensory-evoked responses in ALM, PLM, and ASH are all decreased during L4/A lethargus and this dampening of activity requires *npr-1* (Choi et al., 2013) (Chapter 2)

2) The arousing effects of *npr-1* are blocked by mutations that decrease sensory responsiveness, such as *tax-4* CNG and *osm-9* TRPV mutations (Choi et al., 2013; Chapter 2)
3) Ablating ASH, DVA, or ASI sensory neurons diminishes the *npr-1* quiescence defect (Chapter 2 and 3)

In *C. elegans* there is an overall decrease in spontaneous neural activity during lethargus, which is reminiscent of activity patterns found during sleep in higher organisms (Schwarz et al., 2011). In *Drosophila*, calcium transients in response to stimuli are decreased in the Kenyon cells of mushroom bodies during sleep. In addition, calcium levels in these cells decline when flies fall asleep and increase when they wake up (Bushey et al., 2015). Meta-analysis of brain activity studies in humans using position emission tomography (PET) and functional magnetic resonance imaging (fMRI) have also shown an overall decrease in activity in thalamic structures and frontal regions of the brain during NREM and REM sleep. However, increased activity in the anterior cingulate was also found, demonstrating that not all brain regions become less active in sleep (Jakobson et al., 2012).

Why is such a complex circuit required to regulate the absence of behavior, or quiescence? A more complex circuit may be advantageous since it allows *C. elegans* to adapt its behavior across a range of circumstances. Animals need to not only sustain the quiescent or aroused states for prolonged periods of time, but also be able to switch between behavioral states rapidly.

Sensory gating is a mechanism for inducing quiescence

To our knowledge, sensory gating by NPR-1 in *C. elegans* is one of the best-understood examples of gating as a mechanism to regulate quiescence. We've found that the dampening of sensory activity in multiple neurons discussed above (ASH, ALM, PLM, PDF-1 expressing ASK neurons) requires *npr-1*. NPR-1 acts cell autonomously to dampen activity during lethargus in ASH (Chapter 2), however *npr-1* is not expressed in mechanosensory neurons (ALM, PLM) suggesting that sensory gating by *npr-1* can act cell non-autonomously, likely through PDF-1 signaling (Choi et al., 2013). Therefore, through both direct and indirect mechanisms, NPR-1 acts to gate sensory perception to promote quiescent behavior in *C. elegans*. Gating of ASH activity during lethargus results in asynchronous activity of downstream interneurons AVA and AVD, which is thought to modulate downstream motor circuits to promote quiescence (Cho and Sternberg, 2014). It would be interesting to test if the synchronous activity of AVA and AVD seen during arousal is controlled by glutamate and neuropeptide release from ASH, as predicted by our results.

Others have shown that *egl-4* PKG is also required for sensory gating during lethargus, as *egl-4* mutants show increased responsiveness to sensory stimuli during lethargus and can be rescued by restoring expression of the gene to sensory neurons (Raizen et al., 2008). Upstream of *egl-4* PKG, Notch signaling regulates L4/A quiescence and arousal threshold in an EGL-4- dependent fashion in ciliated sensory neurons (Singh et al., 2011). Understanding how these signaling pathways diminish the sensitivity of various sensory modalities will further our understanding of sensory gating as a mechanism for inducing quiescence.

Less is known about how sensory gating is regulated in mammals, where flow of sensory information is primarily controlled by the thalamus. Thalamic neurons receive input from multiple areas, including the sensory organs, and show distinct states of activity to control state-dependent gating of sensory information during sleep and wakefulness. During wakefulness, thalamocortical relay neurons projecting to sensory cortical areas show tonic activity of fast action potentials, while during sleep these cells are hyperpolarized and show rhythmic bursts of low-threshold calcium potentials (Coulon et al., 2011). Interestingly, NPY reduces activity of retricular thalamic neurons, thereby suppressing thalamic network oscillations that are relevant to sleep and arousal (Sun et al., 2003). Thus, NPY receptors in mammals may also modulate sensory inputs through sensory gatekeeping neurons just as NPR-1 does in *C. elegans*.

Phylogenic parallels in neuropeptide regulation of behavioral arousal

Our studies provide evidence that arousal mechanisms are conserved across phylogeny. As shown in Table 1.1, a number of signaling pathways are conserved across mammalian sleep, Drosophila 'rest', and *C. elegans* lethargus. Most significant to our studies, just as NPR-1 promotes lethargus quiescence in *C. elegans*, NPY in mammals promotes sleep as studies have shown that intravenous injection of NPY in young men enhances sleep quality and reduces the time it takes for subjects to get to sleep (Antonijevic et al., 2000). In addition to the factors mentioned in Table 1.1, here we have shown that glutamate and the orexin-like peptide and receptor, FLP-2 and FRPR-18, regulate quiescence in *C. elegans*. While the role of orexin in sleep is well established,

much less is known about the role of glutamate in arousal. *C. elegans* provides us with a powerful genetic system to study the role of these factors.

Several parallels between orexin signaling in mammals and FLP-2/FRPR-18 in *C. elegans* are notable. First, orexin signaling in mammals can act by a positive feedback loop, with local orexin release activating orexin-expressing neurons through orexin type 2 receptors (Yamanaka et al., 2010). In *C. elegans*, FLP-2 acts similarly, activating FRPR-18 receptors on FLP-2-expressing ASI neurons (Chapter 3). Second, orexin expressing neurons are inhibited by NPY, just as NPR-1 inhibits FLP-2 secretion (Fu, 2004) (Chapter 3). Third, mammalian orexin activates a wide variety of neurons with arousing neurotransmitters (e.g cholinergic, histaminergic, noradrenergic, and serotonergic neurons) (Brown et al., 2012). A number of the cells we identified to express FRPR-18 express similar neurotransmitters (Table 4.1) and are known to promote locomotion (Flavell et al., 2013; Gray et al., 2005; Hu et al., 2011; Li et al., 2006; Piggott et al., 2011). Activation of FRPR-18 by FLP-2 may provide global activation of locomotion promoting neurons in *C. elegans* as a mechanism for arousal.

Table 4.1 Mammalian orexin and *C. elegans* FLP-2 activate neurons with similar arousing neurotransmitters

Mammalian Neuronal Types Activated by Orexin	**Corresponding *C. elegans* neurons expressing FRPR-18 receptor**
Serotonergic	VC4,VC5 (Waggoner et al., 1998)
Histaminergic/Noradrenergic	RIM (Tyramine in *C. elegans* is the closest analog to mammalian NE and histamines) (Alkema et al., 2005)
Cholinergic	AIY, RIM, URA, VC, DVA

Areas of Future Investigation

Sensory modalities that influence behavioral arousal

Our findings show that heightened sensory activity in the RMG circuit causes aroused locomotion both during lethargus and as adults in *npr-1* mutants (Choi et al., 2013) (Chapter 2). As discussed, sensory neurons in the RMG circuit respond to a variety of environmental cues of both positive and negative valence. These include pheromones, oxygen, and aversive odors and chemicals (Fig. 1.1) (Cheung et al., 2005; Gray et al., 2004; Macosko et al., 2009; Reddy et al., 2009). We've also shown that sensory neurons outside of the apparent RMG circuit influence the state of arousal in *C. elegans*, such as the chemosensory neuron, ASI (Chapter 3). An unanswered question is whether the sensory gate-keeping role of NPR-1 applies to all sensory neurons and modalities. One hypothesis is that during molting-associated quiescence, there is a decrease in neural activity in some sensory neurons while an increase in others that is dependent on the sensory valence of the neuron.

One approach to address these questions is to specifically stimulate each sensory neuron with known stimulants during and after lethargus, and see if stimulation influences the animal's state of arousal. Sensory neurons and modalities could then be grouped by which increase quiescence in lethargus vs. those that decrease quiescence. We can then ask if these effects require NPR-1 sensory gating. We have already shown that stimulation of ASH by the aversive chemicals copper and glycerol is decreased during lethargus in wild type worms, and this effect requires *npr-1* (Chapter 2). It would be interesting to see if the same is true for other neurons, such as ASK, which can be

stimulated by ascarosides/pheromones, URX by oxygen, ASI by food (OP50 bacteria), and ADL and AWB by aversive odors such as 2-nonanone.

Indeed, we already have evidence that sensory modalities other than those regulated by ASH are required for arousal from lethargus. First, oxygen likely contributes positively to arousal from lethargus. Inactivation of the soluble guanylate cyclase that binds oxygen, *gyc-35*, suppressed the *npr-1* quiescence defect (Choi, 2013). This correlates with the fact that a decrease in ambient oxygen is likely the reason that the *npr-1* lethargus quiescence defect is diminished in microfluidic devices (Nagy et al., 2014a; 2014b). Overall, these results suggest that ambient oxygen increases arousal.

In addition to oxygen, my preliminary results suggest that pheromones contribute to arousal. *C. elegans* produce a diverse set of ascarosides, the equivalent of mammalian pheromones. Ascarosides are small glycolipids of which there have been over 100 identified in *C. elegans*. These pheromones are sensed by chemosensory neurons (including ADF, ASG, ASI, ASJ, ASK) and have been shown to be involved in a variety of behaviors, including male-hermaphrodite attraction, aggregation, and entry into dauer (Ludewig and Schroeder, 2013). Interestingly, loss-of-function mutations in *npr-1* have been shown to affect hermaphrodite responses to certain ascarosides (Macosko et al., 2009). To test if ascarosides are required for arousal from lethargus, we tested the effect of inactivating the ascarocide biosynthesis genes, *dhs-28* and *daf-22*. These mutants completely lack wild type ascarosides (Butcher et al., 2009; Ludewig and Schroeder, 2013). Inactivating either *dhs-28* or *daf-22* in *npr-1* mutants partially blocked the *npr-1* quiescence defect, suggesting that ascarosides (one or more) are required for arousal from lethargus (Fig. 4.1). Future experimentation will address which ascaroside receptors are

contributing to arousal by inactivating putatative pheromone receptor genes such as *srbc-64*, *srbc-66*, *srg-36*, and *srg-37* (Ludewig and Schroeder, 2013). By narrowing down which receptors are required, we can then determine which specific ascarosides arouse *C. elegans* and on which cells they act.

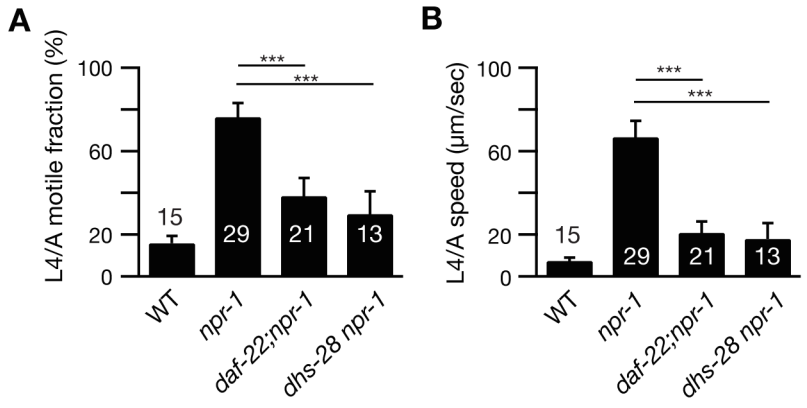

Figure 4.1 Ascaroside biosynthesis is required for the *npr-1* lethargus defect. Locomotion behavior of single worms during the L4/A lethargus was recorded for 30-60 seconds and velocity was measured (2 Hz sampling). Average motile fraction (A) and average locomotion velocity (B) are plotted. The *npr-1(ky13)* L4/A locomotion quiescence defect was suppressed by mutations in *daf-22(ok693)*II or *dhs-28(hj8)*X ascaroside biosynthetic genes. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p<0.001$).

Role of Octopamine and Tyramine in Lethargus

In humans, noradrenergic neurotransmission has been implicated in the regulation of sleep (Brown et al., 2012). While *C. elegans* lack norephinephrine and epinephrine, they do contain two related transmitters, octopamine (OA) and tyramine (TA). Several findings suggest that OA and TA may also regulate arousal in the worm:

1. OA has been shown to inhibit ASH responses (Mills et al., 2011). We have shown that ASH responses promote arousal (Chapter 2).
2. The octopamine receptor *octr-1* and tyramine receptors *tyra-2* and *tyra-3* were amongst the hits in the previously mentioned RNAi screen as suppressors of *npr-1* (data not shown).
3) In *Drosophila*, the biosynthetic enzyme for TA production is rhythmically expressed in clock (PDF expressing) cells and mutants exhibit arrhythmic circadian behavior in locomotion (Huang et al., 2013).

To test if tyramine and octopamine regulate molting-associated quiescence in *C. elegans*, we examined the effect of inactivation of the octopamine biosynthesis pathway genes, tyrosine decarboxylase (*tdc-1*) and tyramine beta hydroxylase (*tbh-1*), on the *npr-1* quiescence defect. In the synthesis of TA and OA, *tdc-1* catalyzes the reaction of tyrosine to tyramine, while *tbh-1* catalyzes the formation of octopamine from tyramine. Therefore, *tdc-1* mutants lack both tyramine and octopamine, while *tbh-1* mutants lack only octopamine. Interestingly, we found that null mutation of *tdc-1* partially suppressed the *npr-1* quiescence defect while inactivation of *tbh-1* had no effect (Fig. 4.2). This result suggests that tyramine is required for the *npr-1* defect and is an arousing neurotransmitter. Consistent with our results, *tdc-1* single mutants were recently reported to have increased total quiescence (duration) and arousal threshold during lethargus (Singh et al., 2014). In contrast, *tbh-1* single mutants were also found to have increased total quiescence and arousal threshold (Singh et al., 2014). TBH-1 and octopamine

synthesis may have a more subtle effect on lethargus that is difficult to discern in the *npr-1* mutant background through our methods.

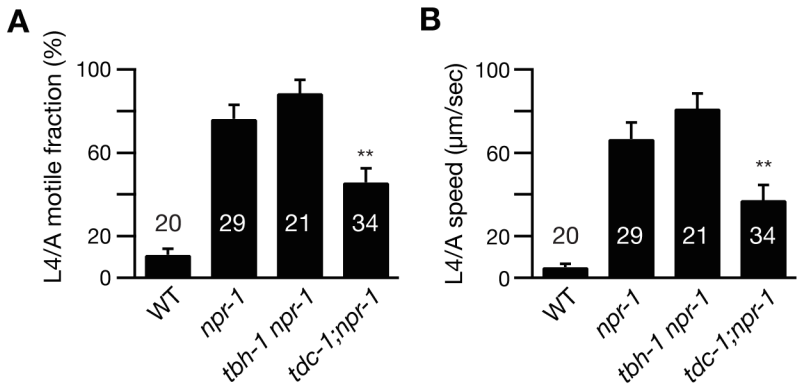

Figure 4.2 Tyramine, but not octopamine, biosynthesis is required for the *npr-1* lethargus defect. Locomotion behavior of single worms during the L4/A lethargus was recorded for 30-60 seconds and velocity was measured (2 Hz sampling). Average motile fraction (A) and average locomotion velocity (B) are plotted. The *npr-1 (ky13)* L4/A locomotion quiescence defect was suppressed by mutations in *tdc-1 (n3420)* II, but not *tbh-1 (n3247)* X. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p<0.001$).

We also tested if exogenous octopamine applied to wild type *C. elegans* has any effect on quiescence. We hypothesized that if octopamine is arousing, it would increase the velocity and motile fraction of animals during lethargus. Consistent with our result that loss of octopamine by *tbh-1* mutation does not suppress *npr-1*, exogenous octopamine had no effect on wild type animals (Fig. 4.3).

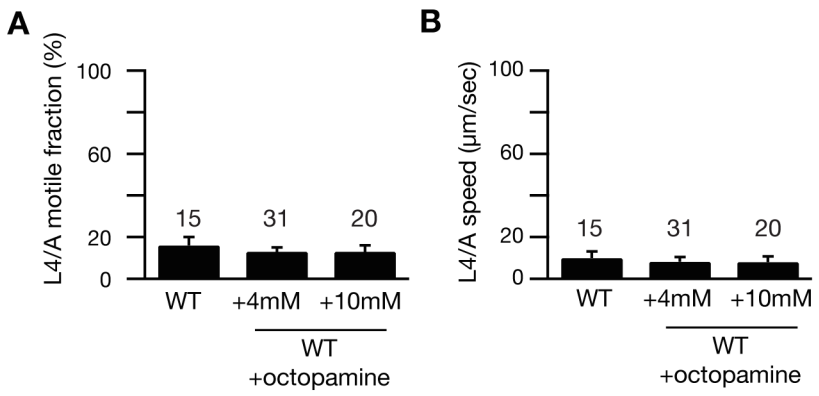

Figure 4.3 Exogenous octopamine has no affect on lethargus quiescence. Locomotion behavior of single worms during the L4/A lethargus was recorded for 30-60 seconds and velocity was measured (2 Hz sampling). Octopamine treated worms were exposed to octopamine in agar plates for 4-5 hr. at the indicated concentrations. Average motile fraction (A) and average locomotion velocity (B) are plotted. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. No values are significantly different.

Future experiments will examine the contribution of tyramine to arousal from lethargus. Which receptors (*tyra-2*, *tyra-3*) are required for *npr-1* quiescence defect? In which cells does tyramine contribute to arousal? Does tyramine act in concert or in parallel with PDF-1 and FLP-2?

Synaptic remodeling as a mechanism for sleep

For a couple reasons, one might propose that structural changes occur at the synaptic or circuit level during quiescence. Synaptic plasticity is thought to be one of the main functions of sleep in mammals. The synaptic homeostasis hypothesis proposes that organisms sleep in order to allow time for restoration of energy stores that are depleted by synaptic strengthening during wakefulness and development (Tononi and Cirelli,

2014). The model states that renormalization occurs via synaptic depression or down-selection during sleep. Synapses are down-selected based on their strength and how often they are activated, promoting memory consolidation and integration (Tononi and Cirelli, 2014). However, recent results suggest that while synaptic homeostasis (or firing rate homeostasis) is gated by behavioral sleep/wake states, it occurs during the waking brain (Hengen et al., 2016). Nonetheless, the depression of synapses during arousal/quiescence may also be a mechanism for reducing circuit activity in sensory neurons or locomotor circuits. Synaptic remodeling as a function of circadian cycles and quiescence has been reported in *Drosophila* and zebrafish. In *Drosophila*, neurons that secrete pigment-dispersing factor show dramatically reduced axonal arborization during nighttime as a function of the circadian clock (Fernández et al., 2008). Interestingly in zebrafish, hypocretin neurons remodel their presynaptic boutons, showing decreased boutons during dark/night cycles in a circadian pattern that is also affected by homeostatic changes in sleep/wake periods (Appelbaum et al., 2010).

Based on these reports, we hypothesized that similar changes may occur during lethargus in *C. elegans*. Indeed, it has been reported that GABAergic synaptic transmission is reduced during lethargus, although no remodeling of UNC-49, the $GABA_A$ receptor subunit, was seen (Dabbish and Raizen, 2011). In finding that GLR-2 AMPA receptors function in DVA to regulate arousal from quiescence (Chapter 2), we asked if any changes occur to GLR-2 synapses in DVA in and out of lethargus. We hypothesized that GLR-2 synapses may be down regulated during lethargus as a mechanism for inducing quiescence. To test this, we visualized GLR-2 synapses in DVA (using the DVA specific promoter, *nlp-12*) by expressing a fusion protein of GLR-2 and

GFP. A punctate pattern in the proximal process of DVA resembled the expected pattern for synapses (Fig. 4.4).

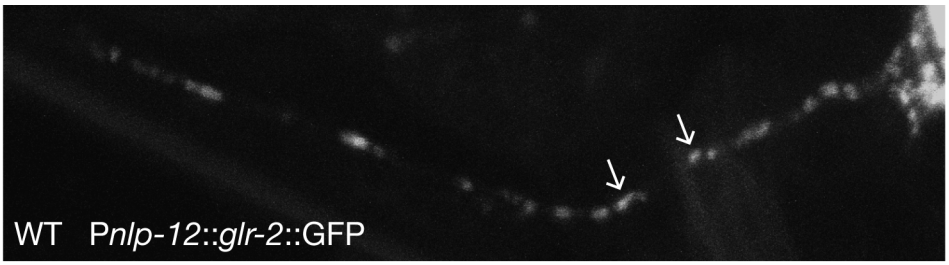

Figure 4.4 GLR-2::GFP shows punctate pattern in DVA proximal process. A construct expressing GLR-2::GFP was expressed in DVA (*nlp-12* promoter) and images taken on a Olympus FV1000 confocal microscope. A punctate pattern could be seen in the DVA neuron proximal process with puncta disappearing as the process entered the cord. Cell body is on far right of image. Arrows indicate example puncta.

We have previously shown that blocking glutamate release increases GLR-1 receptor abundance at synapses (Grunwald et al., 2004). To determine if the GLR-2 puncta in DVA we visualized represented synapses, we tested if they similarly responded to a loss of glutamate release. Indeed, blocking glutamate release by inactivating the vesicular glutamate transporter, *eat-4*, increased GLR-2 receptor abundance (quantified by puncta intensity), suggesting that these puncta were in fact glutamatergic synapses (Fig. 4.5).

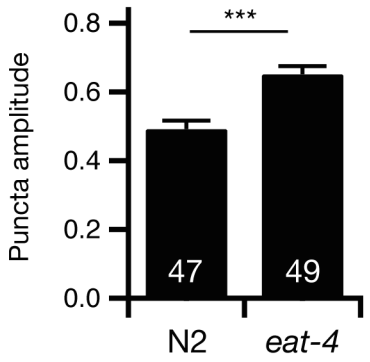

Figure 4.5 Blocking glutamate transmission increases GLR-2 receptor abundance in DVA. Inactivating glutamate release by mutation in the vesicular glutamate transporter, *eat-4*, increased the amplitude of GLR-2:GFP puncta intensity in the DVA proximal process, a pattern consistent with that seen for GLR-1 synapses (Grunwald et al., 2004). GLR-2:GFP was expressed in DVA (*nlp-12* promoter) and analyzed for the indicated genotypes. Images were taken on an Olympus FV1000 confocal microscope. Line scans of the proximal DVA process were analyzed in Igor Pro (WaveMetrics) using custom designed software as described (Dittman and Kaplan, 2006). Values that differ significantly are indicated (***, $p<0.001$).

However, contrary to our hypothesis, we found that there were no changes in GLR-2 synapses before, during, or after lethargus (Fig. 4.6) in wild type of *npr-1* mutant animals. We also tested if expression levels of *glr-2* mRNA changed in and out of lethargus. Interestingly, *glr-2* levels are increased approximately two-fold during the L4/A molt in both wild type and *npr-1* mutants (Fig. 4.7). However, due to the amount of time it takes for a newly transcribed receptor to be delivered to the plasma membrane (5-10 hours) (Greger et al., 2002; 2003), we suspect that newly translated GLR-2 receptors are unlikely to be active until long after the molt, during the adult stage.

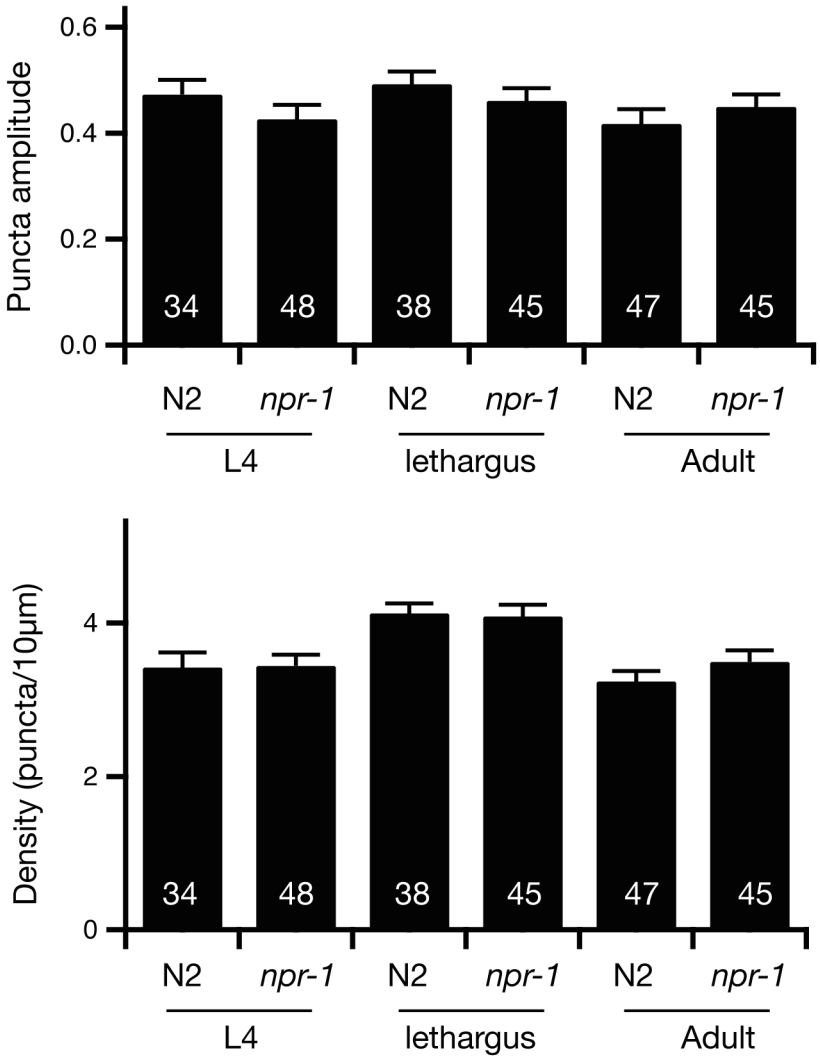

Figure 4.6 GLR-2 receptor abundance and synapse density in DVA is unchanged in and out of lethargus. GLR-2:GFP was expressed in DVA (*nlp-12* promoter) and analyzed for the indicated genotypes and stages. Images were taken on an Olympus FV1000 confocal microscope. Line scans of the proximal DVA process were analyzed in Igor Pro (WaveMetrics) using custom designed software as described (Dittman and Kaplan, 2006). No values are significantly different.

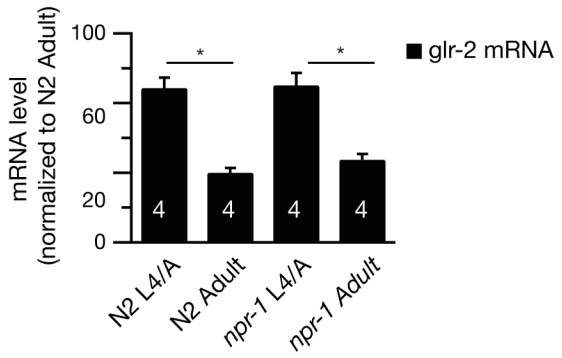

Figure 4.7 *glr-2* mRNA is increased during lethargus. The abundance of *glr-2* mRNA in worm extracts was analyzed by quantitative PCR. Values reported were normalized to those observed in wild type adults. The abundance of *glr-2* mRNAs in lethargus was significantly higher than in young adults. 4 biological replicates were analyzed for each genotype and mRNA. Shown is one primer set for *glr-2* mRNA, although a second showed the same result. Error bars indicate SEM. Values that differ significantly are indicated (*, $p<0.05$).

To conclude, although we have no evidence that synaptic remodeling occurs at GLR-2 synapses during lethargus, we cannot eliminate the possibility that changes are occurring at glutamatergic synapses or others. It will be interesting to assess if synaptic remodeling is occurring in other neurons that have been implicated in quiescence in *C. elegans*, such as the RMG circuit, ASI, or touch neurons. For example, do PDF-1 expressing neurons show plasticity changes as *Drosophila* PDF-positive neurons do? Does ASI show comparable differences to hypocretin expressing neurons in zebrafish?

Role of the RMG circuit

Biological rhythms are governed by inherent timekeeping mechanisms, referred to as circadian clocks. The neurons that regulate these circadian rhythms are typically referred to as clock or pacemarker neurons. In mammals, neurons in the suprachiasmatic nucleus (SCN) are the primary clock neurons, while in *Drosophila* they include the lateral neurons of the central nervous system (Helfrich-Förster, 2004; Maywood et al., 2007; Renn et al., 1999). The molecular basis of the circadian clock is a set of proteins that act by feedback loops on the transcriptional and translational level, resulting in self sustained circadian oscillations (Helfrich-Förster, 2004; Ko and Takahashi, 2006). Our findings highlight a key role of the RMG circuit in regulating arousal in *C. elegans* (Fig. 1.1). We hypothesize that the RMG circuit may function similarly to clock neurons identified in other animals. Previous evidence suggests that lethargus is in fact controlled by clock genes, such as the *C. elegans* homolog of the fly gene PERIOD, *lin-42*. In *C. elegans*, *lin-42* shows rhythmic expression peaking during larval molts (Monsalve et al., 2011). Interestingly, *lin-42* has also been found to regulate the cyclical expression of the quiescence-promoting peptide, NLP-22 (Nelson et al., 2013). In addition, just as the pacemaker lateral neurons secrete PDF in Drosophila, the primary source of *pdf-1* in *C. elegans* is from neurons in the RMG circuit (RMG and ASK) (Fig. 1.1) (Choi et al., 2013; Parisky et al., 2008; Renn et al., 1999). To further explore if the RMG circuit functions similarly to a *C. elegans* clock, we propose testing the following:

1) To test if *lin-42* shows cyclical expression within RMG neurons, as it does in hypodermal cells, we will examine a *lin-42* reporter in the RMG circuit.

2) Findings in *Drosophila* suggest that PDF can influence stability of the period protein (PER) in clock neurons (Li et al., 2014). We will test if mutations in *pdf-1* similarly influence *lin-42* expression, in or outside of the RMG circuit, as a mechanism for regulating locomotion arousal from lethargus. We can similarly test the effects of inactivating *flp-2* and other neuropeptides associated with arousal and quiescence.

3) To test if activity of RMG is altered with respect to quiescent and aroused behaviors, we will examine calcium responses in RMG using a genetically encoded calcium indicator. This experiment will ideally be done in freely moving animals as they enter and exit molting associated quiescence.

While circadian clocks have been primarily associated with 24-hr cycle in other animals (rodents, *Drosophila*), these experiments would explore the role of PERIOD/*lin-42* and clocks that operate on an ultradian periodicity. Additionally, they may demonstrate that clocks can function outside of a light-dark cycle and instead incorporate other sensory inputs.

Concluding remarks on future directions

The experiments proposed and initiated in this chapter advance our work toward better understanding the molecular mechanisms governing the regulation of arousal in *C. elegans.* Follow up experiments on the role of FLP-2 and FRPR-18 in *C. elegans* can provide additional support to our notion that orexin signaling and circuit motifs regulating arousal are conserved in *C. elegans*. Further exploration of how sensory modalities, such as ascarosides, regulate arousal will enhance our understanding of how sensory valence alters circuit activity and behavioral outputs. Examining the role of octopamine and/or tyramine as well as synaptic remodeling will allow continued dissection of the mechanisms controlling quiescence. Last, characterization of the RMG circuit may shed light on the role of circadian timing in *C. elegans* arousal. Altogether, these studies will greatly enhance our understanding of the neuropeptide regulation and sensory-evoked alteration of neural circuits and behavior.

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Appendix A

RNAi and Mutant Screens for Identification of Neuropeptides Regulating Lethargus Locomotion Behavior

Kelsey Taylor and Seungwon Choi performed the RNAi and mutant screens discussed in this appendix during Kelsey's rotation in the lab. A portion of the screen results were published in Choi S, Chatzigeorgiou M, Taylor KP, Schafer WR, Kaplan JM (2015) Analysis of NPR-1 Reveals a Circuit Mechanism for Behavioral Quiescence in *C. elegans*. Neuron 78(5), 869-880.

npr-1 suppressor RNAi screen

To identify other neuropeptides that may regulate behavioral quiescence, we performed an RNAi screen for additional neuropeptide genes whose inactivation suppresses the *npr-1* lethargus locomotion defect. We screened 113 neuropeptide genes including 30 *flp* (FMRFamide-related peptides), 39 *ins* (Insulin-like peptides), 43 *nlp* (non-insulin, non-FMRFamide-related peptides) genes, and *pdf-1*, and used *egl-3* RNAi as a positive control. The RNAi screen was performed as described below. In the primary screen, 16 genes including *pdf-1* were identified whose inactivation suppressed the *npr-1* lethargus locomotion defect significantly more than did the empty vector control (L4440) ($p < 0.05$, Chi-square test) (Table A.1). To validate the results from the primary screen, we subjected 11 out of the 16 positive genes to the secondary screen. We confirmed that inactivation of 5 neuropeptide genes caused significant suppression of the *npr-1* lethargus locomotion defect compared to empty vector controls (Table A.2). The positive genes include *flp-8*, *nlp-5*, *nlp-10*, *flp-33*, and *pdf-1*. Further analyses on double mutants with *npr-1* mutations should confirm the RNAi screen results.

Table A.1 *npr-1* suppressor RNAi screen: Lethargus locomotion behavior (Primary Screen). RNAi was carried out using an RNAi hypersensitive strain in *npr-1* mutant background (*npr-1 nre-1 lin-15b*). 113 neuropeptide genes were screened. After 2 generation RNAi treatment, worms in lethargus (determined by cessation of pharyngeal pumping) were scored as 'wild type-like' (no or little movement) or '*npr-1*-like' (significant movement). 16 genes were identified whose inactivation suppressed the *npr-1* lethargus locomotion defect significantly more than did the empty vector control (L4440) ($p<0.05$, Chi-square test).

Gene	Wild type-like (%)	npr-1-like (%)	Gene	Wild type-like (%)	npr-1-like (%)	Gene	Wild type-like (%)	npr-1-like (%)
nlp-31	3.45	96.55	*ins-1*	28.57	71.43	*flp-12*	40.74	59.26
flp-18	5.88	94.12	*ins-17*	28.57	71.43	*ins-36*	40.74	59.26
ins-33	6.67	93.33	*ins-19*	29.41	70.59	*flp-4*	40.91	59.09
ins-12	9.09	90.91	*flp-3*	29.41	70.59	*nlp-14*	41.38	58.62
flp-19	9.09	90.91	*flp-17*	30.00	70.00	*nlp-15*	41.94	58.06
nlp-26	12.00	88.00	*flp-21*	30.00	70.00	***nlp-38***	42.50	57.50
ins-37	13.89	86.11	*ins-20*	30.00	70.00	*flp-16*	42.86	57.14
ins-29	14.81	85.19	*ins-8*	30.77	69.23	*flp-20*	42.86	57.14
nlp-35	15.63	84.38	*ins-39*	30.77	69.23	*nlp-2*	42.86	57.14
ins-6	15.79	84.21	*nlp-30*	30.95	69.05	*nlp-6*	43.48	56.52
nlp-28	16.67	83.33	*nlp-36*	31.03	68.97	*nlp-7*	43.48	56.52
ins-26	16.67	83.33	*nlp-24*	31.25	68.75	*nlp-17*	44.00	56.00
flp-11	17.24	82.76	*ins-28*	31.43	68.57	*flp-23*	44.44	55.56
ins-27	17.86	82.14	*nlp-46*	31.58	68.42	***ins-5***	44.74	55.26
ins-30	17.86	82.14	*nlp-29*	31.58	68.42	*flp7*	44.83	55.17
nlp-32	17.95	82.05	*nlp-3*	32.14	67.86	*nlp-9*	45.00	55.00
ins-22	18.42	81.58	*ins-32*	32.14	67.86	*ins-16*	45.45	54.55
ins-21	18.75	81.25	*flp-1*	32.26	67.74	*flp-28*	46.43	53.57
ins-31	19.05	80.95	*nlp-39*	32.43	67.57	***nlp-25***	46.67	53.33
flp-26	20.00	80.00	*flp-5*	33.33	66.67	*nlp-11*	46.88	53.13
ins-14	20.59	79.41	*ins-25*	33.33	66.67	*nlp-1*	47.06	52.94
flp-27	21.05	78.95	*flp-15*	33.33	66.67	*nlp-16*	47.06	52.94
ins-18	21.74	78.26	*flp-25*	33.33	66.67	***ins-4***	47.22	52.78
flp-24	22.22	77.78	*nlp-40*	33.33	66.67	*flp-6*	47.83	52.17
ins-23	22.45	77.55	*nlp-33*	34.15	65.85	*nlp-13*	48.28	51.72
daf-28	22.73	77.27	*ins-35*	34.21	65.79	*flp-2*	48.65	51.35
ins-3	22.86	77.14	*nlp-34*	34.48	65.52	***flp-8***	50.00	50.00
nlp-27	23.68	76.32	*nlp-20*	35.29	64.71	***nlp-10***	52.00	48.00
L4440	23.83	76.17	*nlp-12*	35.48	64.52	***ins-9***	52.00	48.00
ins-38	24.14	75.86	*ins-10*	36.36	63.64	***flp-13***	53.33	46.67
flp-14	25.00	75.00	*nlp-21*	36.36	63.64	***flp-32***	54.17	45.83
ins-24	25.00	75.00	*ins-34*	36.84	63.16	***nlp-47***	55.17	44.83
ins-13	25.93	74.07	*nlp-8*	37.50	62.50	***pdf-1***	57.78	42.22
nlp-23	26.47	73.53	*ins-11*	37.50	62.50	***nlp-4***	62.50	37.50
flp-10	26.67	73.33	*nlp-22*	37.93	62.07	***nlp-41***	63.89	36.11
flp-22	26.67	73.33	*ins-2*	37.93	62.07	***nlp-18***	65.71	34.29
nlp-42	28.21	71.79	*flp-9*	38.71	61.29	***flp-33***	70.59	29.41
nlp-19	28.57	71.43	*ins-15*	38.89	61.11	***nlp-5***	77.78	22.22
						egl-3	86.56	13.44
L4440: Empty vector control			*egl-3*: Positive control			*Positive hits: p < 0.05*		

Neuropeptide Mutant Screen

In addition to the RNAi screen discussed above, we also conducted an unbiased mutant screen for neuropeptide genes whose inactivation causes a decrease in lethargus quiescence. *C. elegans* mutant strains were available for more than half of ~120 identified neuropeptide genes. In the primary screen, 68 neuropeptide mutants were screened, and only 1 (*flp-1*) out of 68 genes was identified to have decreased lethargus quiescence significantly more than wild type controls ($p<0.05$, Chi-square test) (Table A.3). However, the effect of *flp-1* mutations on lethargus quiescence was rather subtle compared to *npr-1* mutations. In addition, the deletion allele (*yn4*) that we used to inactivate *flp-1* in the screen also inactivates a neighboring gene, *daf-10* (intraflagellar transport complex component). Thus, further analyses on a new allele that specifically inactivates *flp-1* should confirm the screen results. In addition, the effect of the remaining untested neuropeptides on lethargus behavior should be further addressed by mutant analysis, CRISPR, or RNAi.

Table A.2 *npr-1* suppressor RNAi screen: Lethargus locomotion behavior (Secondary screen). 11 positive genes from the primary screen were subjected to the secondary screen. Inactivation of 5 neuropeptide genes caused significant suppression of the *npr-1* lethargus locomotion defect compared to empty vector controls (L4440) ($p<0.05$, Chi-square test).

Gene	Wild type-like (%)	*npr-1*-like (%)	P value
nlp-4	16.67	83.33	0.404516
L4440	29.03	70.97	1
ins-9	41.94	58.06	0.288358
nlp-18	44.44	55.56	0.223135
nlp-41	47.62	52.38	0.172026
flp-32	50.00	50.00	0.093755
nlp-47	53.13	46.88	0.05215
flp-8	60.00	40.00	0.014916
nlp-5	64.71	35.29	0.016502
nlp-10	72.22	27.78	0.003387
egl-3	77.27	22.73	0.000537
flp-33	79.31	20.69	9.58E-05
pdf-1	100.00	0.00	5.15E-05
L4440: Negative control		*egl-3*: Positive control	
Positive hits: p < 0.05			

Table A.3 Neuropeptide mutant screen: Lethargus locomotion behavior.

68 neuropeptide mutants were screened, and worms in lethargus (determined by cessation of pharyngeal pumping) were scored as 'wild type-like' (No or little movement) or '*npr-1*-like' (significant movement). Only 1 (*flp-1*) out of 68 genes was identified whose inactivation decreased lethargus quiescence significantly more than wild type controls ($p<0.05$, Chi-square test).

Gene	Wild type-like (%)	npr-1-like (%)	Gene	Wild type-like (%)	npr-1-like (%)	Gene	Wild type-like (%)	npr-1-like (%)
Wild type	100.00	0.00	*nlp-12*	100.00	0.00	*flp-6*	100.00	0.00
ins-29	100.00	0.00	*ins-13*	100.00	0.00	*flp-7*	100.00	0.00
nlp-38	100.00	0.00	*flp-27*	100.00	0.00	*flp-11*	100.00	0.00
ins-6	100.00	0.00	*flp-14*	100.00	0.00	*ins-35*	100.00	0.00
nlp-18	100.00	0.00	*flp-18*	100.00	0.00	*nlp-3*	100.00	0.00
ins-33	100.00	0.00	*flp-3*	100.00	0.00	*flp-28*	100.00	0.00
nlp-24	100.00	0.00	*nlp-1*	100.00	0.00	*flp-15*	100.00	0.00
ins-7	100.00	0.00	*flp-24*	100.00	0.00	*ins-8*	100.00	0.00
nlp-35	100.00	0.00	*ins-28*	100.00	0.00	*ins-31*	97.56	2.44
flp-20	100.00	0.00	*nlp-7*	100.00	0.00	*ins-16*	97.37	2.63
ins-18	100.00	0.00	*ins-14*	100.00	0.00	*flp-2*	97.30	2.70
ins-4	100.00	0.00	*ins-27*	100.00	0.00	*nlp-20*	97.22	2.78
ins-19	100.00	0.00	*flp-8*	100.00	0.00	*nlp-36*	97.14	2.86
ins-23	100.00	0.00	*nlp-23*	100.00	0.00	*ins-20*	96.15	3.85
ins-38	100.00	0.00	*flp-25*	100.00	0.00	*flp-21*	96.15	3.85
ins-26	100.00	0.00	*flp-5*	100.00	0.00	*nlp-15*	96.15	3.85
ins-22	100.00	0.00	*ins-3*	100.00	0.00	*nlp-29*	95.65	4.35
ins-30	100.00	0.00	*ins-11*	100.00	0.00	*nlp-5*	93.75	6.25
ins-9	100.00	0.00	*flp-33*	100.00	0.00	*flp-26*	93.33	6.67
ins-34	100.00	0.00	*flp-16*	100.00	0.00	*flp-23*	92.86	7.14
nlp-9	100.00	0.00	*flp-17*	100.00	0.00	*flp-10*	92.59	7.41
nlp-14	100.00	0.00	*flp-19*	100.00	0.00	*daf-28*	85.71	14.29
flp-9	100.00	0.00	*flp-12*	100.00	0.00	***flp-1***	**81.48**	**18.52**
						npr-1	13.48	86.52
Wild type: Negative control			*npr-1*: Positive control			*Positive hits: p < 0.05*		

Materials and Methods

Strains

KP6050 *npr-1(ky13) nre-1(hd20) lin15b(hd126)* X

RNAi feeding screen

A small-scale RNAi feeding screen was performed as described (Kamath et al., 2003). The screen was performed in the neuronal RNAi hypersensitive mutant background (*nre-1 lin-15b*) (Schmitz et al., 2007). 15 neuropeptide genes known to be expressed in RMG circuit were selected for the screen (Li and Kim, 2008). After 5 days of RNAi treatment (2 generation) at 20°C, well-fed late L4 animals were transferred to full lawn OP50 bacterial plates. After 1 hour, animals in lethargus (determined by absence of pharyngeal pumping) were scored for their motility. Statistical significance was determined using chi-square test.

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